UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

GRANT APPLICATION

OMB Approved 0524-0022
Expires 6/92

FOR CSRS USE ONLY	
Program Area Code	Proposal Code

1. Legal Name of Organization to Which Award Should be Made	3. Name of Authorized Organizational Representative	4. Telephone Number (Include Area Code)
Iowa State University	Mr. R.E. Hasbrook	R

2. Address (Give complete mailing address and Zip Code-Including county)	5. Address of Authorized Organizational Representative (If different from Item 2.)
209 Beardshear Hall Iowa State University Ames, Iowa 50011	same

6. Title of Proposal (80-character Maximum, including spaces)

Auxin Regulation of Proteinase Inhibitor Genes in Transgenic Tobacco

7. Program to Which You are Applying (Refer to Federal Register Announcement where applicable)	8. Program Area and Number (Refer to Federal Register Announcement where applicable)
22.0 Atmosphere and Global Climate Change	22.1 Plant Responses to the Environment

9. IRS No.	10. Congressional District	11. Period of Proposed Project Dates	12. Duration Requested
R	Iowa 4	From: 07/01/92 Through: 06/30/95	24 months

13. Type of Request

☒ New ☐ Resubmission ☐ Renewal ☐ Continuing Grant Increment
☐ PI Transfer ☐ (of/to USDA Grant No. _____) ☐ Other

14. Funds Requested (From Form CSRS-55

REDACTED

15. Principal Investigator(s)/Project Director(s) a. PI/PD #1 Name (First, Middle, Last) and Social Security Number* (Correspondent PI)	16. PI/PD #1 Phone Number (Include Area Code)
Robert Thornburg R	Phone: REDACTED FAX:
b. PI/PD #2 Name (First, Middle, Last) and Social Security Number	16. PI/PD #1 Business address (Include Department/Zip Code)
	Robert Thornburg
c. PI/PD #2 Name (First, Middle, Last) and Social Security Number	REDACTED

Submission of the Social Security Number is voluntary and will not affect the organization's eligibility for an award. However, it is an integral part of the CSRS information system and will assist in the processing of the proposal

18. Type of Performing Organization (Check one only)

- 01 ☐ USDA/S&E Laboratory
- 02 ☐ Other Federal Research Laboratory
- 03 ☐ State Agricultural Experiment Station (SAES)
- 04 ☒ Land Grant University 1862
- 05 ☐ Land Grant University 1890 or Tuskegee University
- 06 ☐ Private University or College
- 07 ☐ Public University or College (Non Land-Grant)
- 08 ☐ Private Profit-making
- 09 ☐ Private Non-profit
- 10 ☐ State or Local Government
- 11 ☐ Veterinary School or College
- 12 ☐ Other (Specify)

19. Will the Work in this Proposal Involve Recombinant DNA?

☐ No ☒ Yes (If yes, complete Form CSRS-662)

20. Will the Work in this Proposal Involve Living Vertebrate Animals?

☒ No ☐ Yes (If yes, complete Form CSRS-662)

21. Will the Work in this Proposal Involve Human Subjects?

☒ No ☐ Yes (If yes, complete Form CSRS-662)

19. Will this Proposal be Sent or has it Been Sent to Other Funding Agencies, Including Other USDA Agencies

☐ No ☒ Yes (If yes, list Agency acronym(s) & program(s))

NSF

By signing and submitting this proposal, the prospective grantee is providing the required certifications set forth in 7 CFR Part 3017, as amended, regarding Debarment and Suspension and Drug-Free Workplace; and 7 CFR Part 3018 regarding Lobbying. Submission of the individual forms is not required. (Please read the Certifications and Instructions included in the kit before signing this form.). In addition, the prospective grantee certifies that the information contained herein is true and complete to the best of its knowledge and accepts as to any grant award, the obligation to comply with the terms and conditions of Cooperative State Research Service in effect at the time of the award.

Signature of Principal Investigator(s)/Project Director(s) (All PI's/PD's listed in block 15 must sign if they are to be included in award document.)	Date
Robert Thornburg	1/30/92
Signature of Authorized Organizational Representative (Same as Item 2)	Title: Contracts/Grants Officer — Date: 1-30-92

Form CSRS-661 (9/89)

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Table of Contents

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Principal Investigator(s) or Project Director(s):

PI/PD #1 Robert Thornburg Institution Iowa State University

PI/PD #2 _____ Institution _____

PI/PD #3 _____ Institution _____

Project Title: Auxin Regulation of Proteinase Inhibitor Genes In Transgenic Tobacco

AREA AWARDS

If you are applying for an AREA Award, check type:

- ☐ Postdoctoral
- ☐ New Investigator
- ☐ Strengthening:
  - ☐ Career Enhan.
  - ☐ Equipment
  - ☐ Seed Grant
  - ☐ Standard Proj.

(Summary must fit in box)

PROJECT SUMMARY

The proteinase inhibitor gene system in solanaceous plants is one of the best characterized inducible gene systems in plants. We have previously prepared transgenic plants that express marker genes under control of the Proteinase Inhibitor II promoter (*pin*2-CAT). We are using these plants to assess the early biochemical events that occur following wounding of the foliage. We have found that indole-3-acetic acid (IAA) represses the expression of the *pin*2 gene system in the foliage of our transgenic tobacco. There are, however, other compounds that are inducers of the *pin*2 genes in plants. In this proposal we will compare each of these inducers with the repressor, IAA, to determine if each of these inducers acts through a single IAA repressible signal transduction pathway, or whether there are several signal transduction pathways involved. We also have preliminary evidence for the involvement of polar auxin transport in the natural induction of the *pin*2 gene system in plants. We will examine this question in some detail, because it provides a single framework for the induction of the *pin*2 genes in plants by all known inducers. Finally, we will assess the role of IAA repression of the *pin*2-CAT gene activation in whole plants. We will make plants that have elevated or reduced levels of IAA and directly examine the effects of altered levels of IAA on wound induction. Our long term goals are to fully understand the molecular and genetic mechanisms involved in the induction of the *pin*2 gene system in plants. Understanding the molecular principles involved in the induction of this late class of wound-inducible genes will aid us in the eventual goal of manipulating crops in the field for a more sustainable agriculture.

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Project Description and Preliminary Results:

Wound-Inducible Proteinase Inhibitor Genes:

Plants respond to wounding by inducing a variety of quiescent gene systems involved in plant defenses. These defense genes have at least two identified functions, to close the wound (Dixon 1986), and produce biochemicals that inhibit the growth of potentially harmful herbivores and invading microorganisms (Ryan 1978).

Different biochemical pathways control these functions and they produce dramatically different compounds. Further, these pathways are regulated differently. The difference in the regulation of these genes is best shown by their kinetics of induction. To close a wound, plants rapidly induce the biosynthesis of lignin in the near vicinity of the wounded plant tissues. The phenylpropanoid pathway is responsible for the production of lignin, phytoalexin antibiotics and flavanoids. Wounding results in the induction of many steps in the phenylpropanoid and related biochemical pathways. Among these steps are the genes encoding phenylalanine ammonia lyase, chalcone synthase, and others. The induced phenylpropanoid genes, plus some others such as chitinase represent one class of wound-inducible genes. These early inducible genes are induced rapidly following wounding. Messenger RNA for the key phenyl propanoid pathway enzymes appears within 15 minutes of a wound (Dixon, 1986; Lawton, et al., 1983; Bevan and Northcote, 1979).

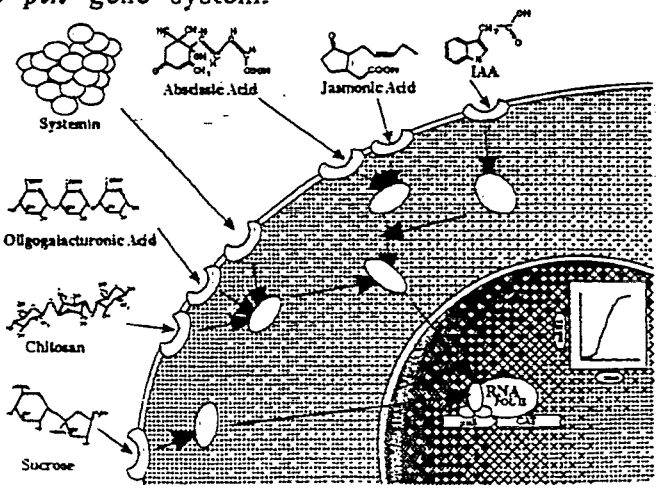
Aside from the genes coding for lignin and phytoalexin production, there are other genes induced later in the wound-cascade. Among this late-inducible class of genes are the proteinase inhibitors. The mRNA for the proteinase inhibitors from solanaceous plants does not appear until several hours after the plant tissues are wounded (Graham, et al., 1986).

These proteinase inhibitors from solanaceous plants (potato and tomato) play a defensive role in plants (Green and Ryan 1972; Ryan 1981). The proteinase inhibitors are expressed in the foliage of the potato and tomato plants, in response to insect attack or mechanical wounding (Green and Ryan 1972; Green and Ryan 1973). These small, proteins are very effective inhibitors of animal and microbial digestive enzymes that limit the nourishment gained by an invading insect or microbe (Ryan.1966).

The cDNAs (Graham et al. 1985a; Graham et al. 1985b; Sanchez-Serrano et al. 1986), and the genes for the wound-inducible proteinase inhibitors have been isolated from both potato and tomato (Lee et al. 1986; Fox 1986; Cleveland et al. 1987; Thornburg et al. 1987; Keil et al. 1986). The PI has identified, isolated, and characterized several members of the Proteinase Inhibitor II (*pin2*) gene family and demonstrated the expression of one of these *pin2* genes in plants in response to wounding and insect attack (Thornburg, et al., 1987a; Thornburg, et al., 1987b; Kernan and Thornburg, 1989; Thornburg, et al., 1990). Further, we have identified sequences in the *pin2* promoter that may regulate the wound-activation of these genes. These sequences are deleted in a natural *pin2* variant that is not wound-inducible.

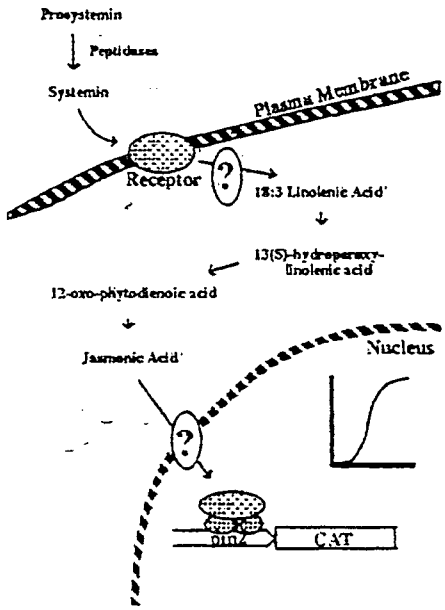
We have constructed chimeric wound-inducible marker genes driven by the wound-inducible *pin2* promoter and transformed tobacco plants with these chimeric genes (Thornburg, et al., 1987a). We are using these transgenic plants that express the *pin2*-CAT gene to explore the mechanisms responsible for the activation of these quiescent genes following wounding of the plant foliage. The underlying molecular mechanisms that control the activation of these proteinase inhibitor (pin) genes in plants however, remain largely unknown. It is our long term goal to elucidate these mechanisms. The inducible proteinase inhibitor gene system is one of the best-studied inducible gene systems in plants. Many different biochemical signals have been identified which can alter the expression of the wound-inducible *pin* gene system.

The figure at right (Appendix 1) shows there are many different biochemicals that can affect the expression of the proteinase inhibitors in solanaceous plants. These include fragments from plant (Bishop, et al., 1981; Bishop, et al., 1984) or fungal cell walls (Walker-Simmons and Ryan, 1984), the general metabolite sucrose (Johnson and Ryan,



1990), and the plant growth regulators abscisic acid (Pena-Cortez, et al., 1989), auxin (Kernan and Thornburg, 1989), acetylsalicylic acid (Doherty, et al., 1988), and jasmonic acid (Farmer and Ryan, 1990). In addition, an 18 amino acid peptide termed systemin that functions to induce the proteinase inhibitor gene system at femtomolar concentrations has recently been isolated from tomato leaves (Pearce, et al., 1991). This list of inducers contains polyanions, polycations, peptides, metabolites and hormones. The physical nature of this diverse group of compounds is amazing for molecules that all induce the same gene and the molecular mechanisms mediating the activation of the wound-inducible genes by these compounds are largely unknown. Presumably, these compounds must be individually recognized by the cell and the signal transduced to the nucleus where transcription of the *pin2* promoter initiates. However, one can propose in which some or all of these very diverse compounds may affect a single physiological response that results in the induction of the *pin2* genes.

Several of the inducing compounds have been recently proposed to act through a signal transduction pathway that results in the activation of the *pin2*-CAT genes (see figure at right). However, many steps in this pathway are still uncharacterized. Further, this scheme does not include all the known indercers of the *pin2* system. The goal of this proposal is to determine where auxin inhibition of the *pin2*-CAT gene fits into this proposed signal transduction scheme and to begin to understand how IAA levels change following wounding in plants.



The long term goals of my laboratory are to understand the basic molecular mechanisms that control induction of a plants defensive genes in response to insect attack. Towards that goal, we have been examining the early biochemical events that occur in tobacco foliage following wounding. From these studies we concluded that IAA levels in plant's foliage correlate with wound-inducibility of the

gene, and that IAA levels change in leaves following wounding. This proposal will answer basic questions of whether IAA does play a central role in the activation of the *pin2* genes in transgenic tobacco.

Role of Auxin in *pin2* Gene Regulation:

Our work with auxin indicates that IAA may play a central role in the expression of the Proteinase Inhibitor II genes

Our early work on the regulation of chimeric genes driven by the *pin2* promoter illustrated that wound-induction of the proteinase inhibitor genes was position specific in plant leaves. Because auxin gradients exist in plants (Schneider and Wightman, 1978) we decided to examine the role of auxin in the regulation of the wound-inducible marker genes.

Briefly, we prepared callus from our transgenic plants and plated these calli on Murashige-Skoog medium containing no auxin. Under these conditions, we observed high levels of expression of the *pin2*-CAT gene in the transgenic callus (Kernan and Thornburg, 1989; Appendix 9). This expression did not require wounding of the callus. Merely by placing the calli on media lacking auxin we could observe the activation of the wound-inducible *pin2*-CAT construction. We then added auxins back to the media to affect CAT expression. These studies showed that addition of auxin to the media inhibits the expression of the CAT protein driven by the wound-inducible *pin2* promoter. This inhibition occurs at near physiological concentrations of auxin in whole plants. This effect of auxin was also inhibitory in whole plants.

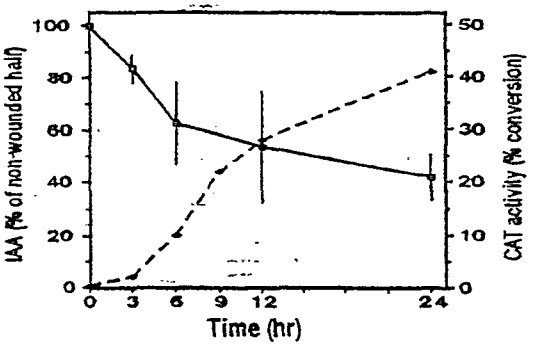
Levels of IAA in Whole Plant Tissues

The fact that exogenously added IAA down-regulates the expression of the *pin2*-CAT gene in plant tissues and in whole plants raises an interesting question. What happens to IAA levels in whole plants following a wound? To address this problem, we examined the levels of IAA in whole plants by isotope dilution techniques using gas chromatography mass spectrometry to detect IAA from whole plant tissues.

To conduct these experiments, we used a half-leaf assay. In this assay we first removed and froze in liquid nitrogen one half of the leaf blade up to but not including the midvein as a time zero control. Then the remaining half of the leaf, still attached to the plant was wounded. After incubating on the plant, we removed the second half-leaf (up to but not

including the midvein) and both samples (unwounded and wounded) were and stored at -70°C until processed. IAA was measured by an isotope dilution technique (Thornburg and Li, 1991; Appendix 10).

The results of some of these studies are shown in Figure 2 (at right; Appendix 2). This figure shows the percentage of IAA remaining in the wounded half of the leaf compared to IAA present in the non-wounded half of the leaf. With time, following wounding, IAA in bulk leaf tissues declines by two to three fold. In addition, the decline in IAA mirrors the kinetics of induction of the *pin*2-CAT construction. While the correlation of induction with the decline in IAA suggests that these observations are related, we cannot yet exclude the possibility that IAA decline may trigger a secondary event that stimulates *pin*2 induction. To elucidate this is one of the goals of this research proposal. We further know that the induction of the *pin*2-CAT gene within a leaf is not uniform throughout that leaf (see figure 4 below; Appendix 4). Therefore, an added goal of this proposal is to measure the change in IAA levels in those parts of a leaf where the induction is occurring.



The exact mechanisms that lead to a decline in IAA are unknown. Free IAA within plant tissues can be influenced by changes at one of several points. These include biosynthesis, degradation, the formation of amide or ester storage forms of IAA, or through IAA transport.

Ways that IAA levels could change:

Because our measurements of IAA determine the pool size at any given point of time, we have not yet determined why IAA levels change. However, the pool size of any biochemical compound can change through changes in its biosynthesis, its degradation or by modification into storage forms. In the shoots of tobacco (Phelps and Sequeira, 1967) and tomato (Gibson, et al., 1972) plants can convert L-tryptophan into IAA by way of tryptamine. The first step of this pathway is catalyzed by tryptophan

decarboxylase. The resulting tryptamine is then oxidatively deaminated to indole-3-acetaldehyde by tryptamine oxidoreductase (Bower, et al., 1976). The indole-3-acetaldehyde is converted into indole acetic acid by one of two enzymes, either NAD-dependent indole acetaldehyde dehydrogenase of by an oxygen requiring indole acetaldehyde oxidase (Goodwin and Mercer, 1983). We have not yet begun to look at the enzymes involved in the biosynthesis of IAA, however, if wounding causes changes in IAA biosynthesis the site of this regulation can be somewhat narrowed. Such regulation must occur after the decarboxylation of tryptophan to tryptamine, because plants that overexpress tryptophan decarboxylase do not show elevated of IAA (Songstad, et al., 1990).

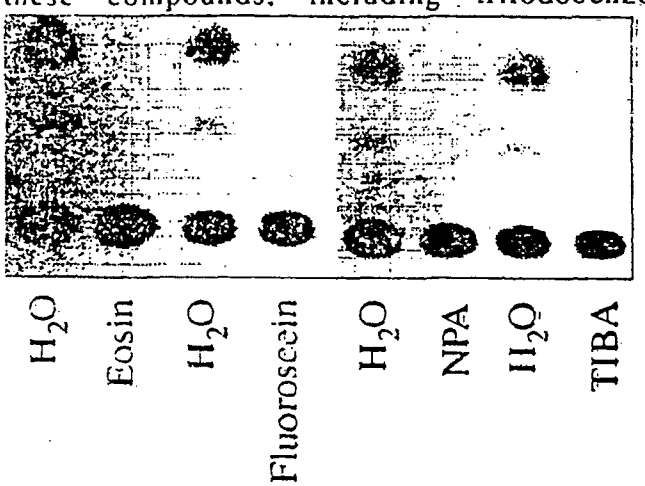
Plants also change free IAA levels in their tissues by covalent attachment of either amino acids (Andreae and Good, 1955, Olney, 1968, Liu, et al., 1978) or carbohydrates (Labarca, et al., 1965, Piskornik and Bandurski, 1972). Such modified IAA is termed bound IAA. We have previously determined that these IAA conjugates are 5 to 10 fold less active than free IAA in regulating *pin2* gene expression (Kernan and Thornburg, unpublished). We are currently examining the levels of IAA conjugates in plants following wounding to determine if wounding causes a shift in free IAA to bound IAA levels.

Biochemical pool sizes can also be affected by catabolism. There are at least two pathways for the degradation of IAA into inactive compounds. The first of these is through the enzyme IAA oxidase that converts IAA into 3-methylene oxindole. This is probably the minor degradation pathway, indeed some questions remain whether this is truly a pathway that functions *in vivo*.

The major pathway for degradation of IAA in whole plants is through the rapid oxidation of IAA into Oxindole-3-acetic acid. There is then a slower addition of a hydroxyl group to the 7 position of OxIAA (Nonhebel, 1982). This 7-hydroxy OxIAA is then glucosylated to form a compound that lacks all auxin activity (Henderson and Patel, 1972, Weiss, 1966). We have not yet begun studies to characterize the levels of the catabolic products of IAA during the wounding process. This is a long term goal and we will eventually seek collaborative help in this aspect of IAA's involvement in the induction of the *pin*2-CAT gene.

IAA Transport Inhibitors Block Wound-induction

One additional aspect of auxin action in plants that needs consideration is auxin transport. To further establish the role of auxin in the wound-inducible activation of the *pin2*-CAT construction, we have used compounds that promote the intracellular accumulation of auxin by inhibiting cellular efflux and transport of auxin. We have used a series of these compounds, including triiodobenzoic acid (TIBA), naphthylphthalamic acid (NPA), fluoroscein, and eosin. Each of these compounds inhibits auxin transport (Katekar and Geissler, 1975; Katekar and Geissler, 1977b; Thompson, et al., 1973). As shown in the figure at left (Appendix 3), each of these compounds inhibits the activation of the *pin2*-CAT gene in our transgenic plants. Thus, compounds that cause intracellular accumulations of IAA within cells can uncouple wounding from the activation of the *pin2*-CAT gene.



Our investigations have, therefore, shown that IAA levels negatively influence the activation the *pin2*-CAT gene system in transgenic callus and whole plants. Further, IAA levels in the leaves of plants correlate with induction of the *pin2*-CAT gene. These levels also decline with a timeframe that is consistent with IAA being a regulator of the *pin2*-CAT gene in these transgenic tobacco plants. In addition, inhibitors of IAA transport likewise inhibit wound-inducible activation of the *pin2*-CAT construction in our transgenic tobacco plants. These findings have led us to conclude that IAA plays a role in the induction of the *pin2*-CAT gene system in whole plants.

Our long term goals are to understand this role of IAA in the activation of the wound-inducible *pin2*-CAT gene in our transgenic tobacco plants and to understand the relationship between each of the above biochemical inducers (Figure 1) and IAA.

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Specific Goals

To increase our understanding of the role of IAA in the regulation of wound-inducible *pin2* expression, we propose to ask four readily approachable questions that relate to this problem:

1) What is the relationship of IAA to the other biochemicals that affect the expression of the wound-inducible *pin2* genes?

2) How much do IAA levels change in the tissues that express the *pin2*-CAT gene?

3) Is IAA transport involved in the regulation of *pin2* gene expression *in vivo*?

4) Does elevating or reducing IAA in whole plants affect their ability to express the *pin2*-CAT gene?

Answering these questions should provide meaningful new information about the mechanisms regulating the expression of the late class of wound-inducible genes.

Experimental

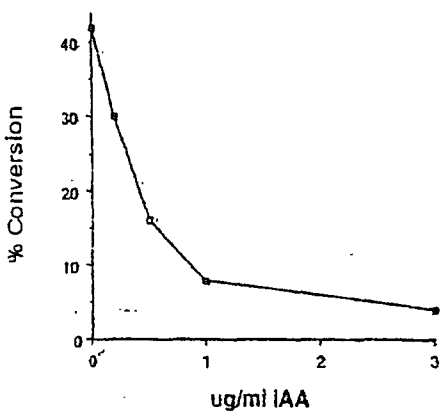
Objective 1) What is the relationship of IAA to the other biochemicals that affect the expression of the wound-inducible *pin2* genes?

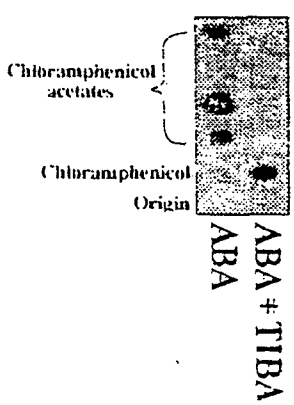
All biochemical effects of wounding will be correlated with the activation of the *pin2*-CAT gene *in vivo* by directly assaying the chloramphenicol acetyl transferase gene in wounded or induced tissues. The plants used in this study were earlier described (Thornburg, et al., 1990). They were an R4 (4 selfings after regeneration, R0), homozygous line of transgenic plants containing a *pin2*-CAT construction that was also earlier described (Thornburg, et al., 1987). When we examined the expression of CAT protein in these plants, we found that expression of the *pin2*-CAT gene modeled to the expression of the proteinase inhibitors in either potato or tomato. We use each plants once and then never again, so that multiple wounds do not occur on the same plant. One previously unforseen boon of our field trials on transgenic plants (Thornburg, et al.,

1990; Thornburg, 1991) is that we how have in hand a large amount of homozygous seed from our R4 transgenic plants (we estimate: 2 lbs of homozygous Tr25 seed at 2 x 10^4 seeds per gram = about 2 x 10^8 seeds). It is therefore, no problem to produce large numbers of near isogenic plants.

Because there are many biochemical compounds that affect the expression of the wound-inducible ***pin2*** genes (see above), we want to sort through these compounds systematically and understand their relationships to each other and to the ***pin2*** activation response. We have begun such a comparative study by comparing of ABA and IAA.

ABA, at very high concentrations, has been reported to induce the wound-inducible ***pin2*** gene in potato and tomato (Pena-Cortez, et al., 1989). We also observe this induction by ABA, but only at extremely high concentrations of ABA (100 µM). When we compare these two compounds directly as shown in the figure at right, we observe that the inhibitory role of IAA is apparently the dominant response that is measured by the plant. Further, the repression of the ABA induced gene response by IAA in these plant tissues occurs at physiological concentrations of IAA. We have also examined the effects of compounds, such as triiodobenzoic acid (TIBA) that inhibit the cellular efflux of auxin, we observe that TIBA also blocks the induction of the ***pin2*** gene system by ABA (see figure at left). Therefore, those compounds that cause intracellular accumulation of IAA within tissues not only block the wound response (see Page 10) but they also block the induction of the gene system by ABA induced turnon. We have made similar observations for the effect of IAA and TIBA on sucrose induced expression of the ***pin2*** gene system. These observations also show that IAA also blocks the sucrose-induced expression of the *pin2* gene system in our transgenic tobacco plants. We





would like to extend these observations to include all the compounds shown in Figure 1.

To begin, we will conduct experiments similar to those described above, except we will substitute the other compounds into the system. Most of these compounds are already in hand. They have been purchased from commercial sources (ABA, IAA, chitosan, sucrose, methyl jasmonate) or have been prepared. Systemin was synthesized in the Iowa State University Protein Analysis Facility on an Applied Biosystems Model 340A peptide synthesizer by standard procedures (see Appendix 4).

We will induce the *pin*2-CAT gene system independently with each of the compounds shown in Figure 1 (Appendix 1). The induction of the *pin*2-CAT gene system will be conducted in the presence and the absence of varying concentrations of IAA. Following a period of incubation, we will then measure the level of CAT activity in these tissues to determine whether IAA can block the induction of the *pin*2-CAT gene system by each of these compounds.

If any inducor is not repressed by IAA at near physiological levels, we will assume induction of *pin*2-CAT gene by a signal transduction pathway that differs from the auxin repressible signal transduction pathway. One of the main endpoints of this objective will be, therefore, to demonstrate how many different signal transduction pathways are effective in regulating the *pin*2 gene system in plants.

Wounding and *in vitro* Induction:

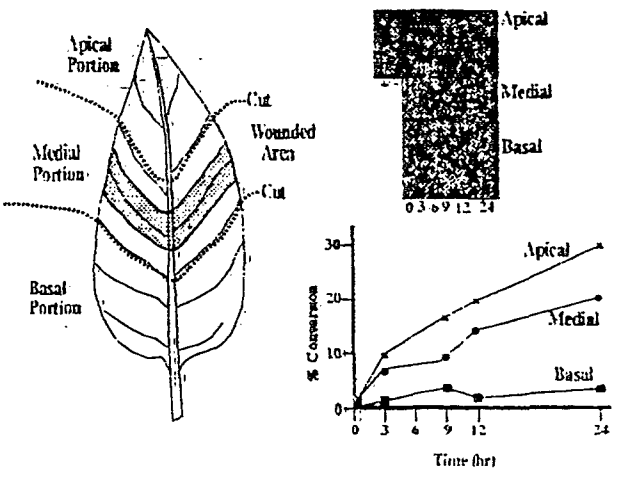
When we assess the wound-induction of our transgenic plants we use only fully expanded leaves. They are wounded by repeated pinching with a pair of surgical hemostats that make a severe wound across the central part of the leaf as earlier described (Thornburg and Li, 1991). After an incubation period of 18 to 24 hours, the leaves are removed and immediately processed for CAT assay. For *in vitro* induction, leaves are sliced from the transgenic plants by making a single clean cut with a razor blade. Leaves accumulate CAT protein following induction when the petiole is placed into a solution of Murashige-Skoog (MS) liquid medium (Murashige and Skoog, 1962) without hormones, but containing 3% sucrose or other inducers. We usually repeat each experiment several times with 6 to 8 leaf samples to insure consistent results.

CAT Assays:

Homogenization of the leaves produces an extracts of transformed tobacco leaves for analysis. Following grinding, the expressed juice is twice centrifuged for 5 minutes at 10,000 x g and the clear supernatant is recovered for protein analysis by the method of Bradford (1976). A quantity from each extract containing 100 ug of protein is assayed for CAT activity using [^{14}C]-chloramphenicol (60 uCi/mmol) as substrate (Gorman, et al., 1982). Following TLC analysis, the activity of the CAT protein is visualized by exposure to x-ray film. The TLC plates are overlaid onto the exposed film and the radioactive spots corresponding to labelled chloramphenicol acetates and unreacted chloramphenicol were cut out and counted in a Packard liquid scintillation counter. This "cutting and counting" of our CAT assays can be severly limiting, because we routinely run several hundred (300-400) CAT assays per month. We have recently investigated the use of a digital video system of data collection and analysis for use with CAT assays. We find that this system reduces the time required for data collection and analysis and we intend to convert our data collection and analyses to this easier method.

Objective 2) <u>How much do IAA levels change in the tissues that express the *pin2*-CAT gene?</u>

Our previous studies have indicated that IAA within a leaf declines by a factor of 2 to 3 and the kinetics of decline are similar to the kinetics of ***pin2*-CAT** activation. These studies compared the wounded and non-wounded leaf halves (see Figure 2; Appendix 2). However, we have also previously shown that the induction of the ***pin2*-CAT** system occurs primarily at the tip of the leaf, and not at the base of the leaf (see Figure 6, below; Appendix 6). This unequal induction of the *pin2*-CAT gene in different parts of a leaf also correlates with the IAA levels in



different parts of a tobacco leaf (Avery, 1935). These studies, together with those presented above, indicate that IAA may be transported from the tip of the leaf downwards. We believe that this movement of IAA creates regions within the leaf where IAA levels decline and this derepresses *pin*2-CAT gene system in those tissues. Therefore, we will directly examine the change in IAA levels in different parts of leaves following wounding.

At several times after wounding (ie, 3 hr, 6 hr, 9 hr, 12 hr, 18 hr, 24 hr and 48 hr) we will analyze our plants for IAA in the leaf parts. We will adapt our current IAA analysis protocol to this end. We will remove a non-wounded leaf half, then divide it into three sections, the apical, the medial, and the basal parts (see Figure 6). Then the leaf half remaining on the plant will be wounded. After the proper incubation period, we then harvest the other half, divide it into the same three parts and freeze each part separately in liquid N_2. Then each of the parts (basal, medial, and apical) of each of the two leaf halves (wounded and non-wounded) will be processed for IAA content and for CAT assay. The data will be expressed as percentage decline in IAA within each parts of the leaf. Because we are dividing these leaves into 6 sections, it may be necessary to process several leaves from a single plant in each sample. Only large fully expanded leaves from the central part of the plant will be grouped together for this analysis.

IAA Analysis:

The method that we are now using to measure levels of endogenous IAA in whole plant tissues, isotope dilution analysis, although a bit cumbersome is extremely exact, and quite reproducible (Pengelly, et al., 1981). This method corrects for differential loss in hormone recovery during purification. It calls for the extraction of IAA from plant tissues following the addition of [^{13}C]-labeled IAA (Cohen, et al., 1986). (The [^{13}C]-IAA standard is uniformly labeled at each of the 6 carbons of the 6 membered ring of IAA. Therefore, the 13C labeled IAA has 6 AMU more than native IAA. The standard has been kindly provided by Dr. Robert Bandurski, Michegan State University.) The extracted IAA is methylated with diazomethane to make it volatile, and partially purified with a Hplc step on a C-18 reverse phase column. The partially purified IAA-methyl

ester is concentrated to a small volume (10 ul) and aliquots are injected into a Hewlet Packard 5890 gas chromatograph. We use a splitless injection; so that 100% of the GC effluent is bled into a Hewlet Packard 5970 quadropole mass spectrometer. This spectrometer uses an electron impact method to generate ionizations. We selectively measure ions at 189 and 195 AMU (the parent peak, IAA minus one electron) and 130 and 136 AMU (the most abundant peak, IAA minus the carboxymethyl group). Because we know how much [^{13}C]-labeled standard IAA we added, we can compare the 185 to 195 peaks and the 130 to 136 peaks to calculate IAA levels present in leases at sampling. These two ion pairs are then correlated with each other to insure that both estimates agree thereby increasing selectivity. With this method we can readily detect 5 ng of IAA that represents less than 1/3 gram of unwounded leaf tissue. In addition, methods are available (Li, et al., accepted for publication) to determine the levels of both ABA and IAA with a single tissue sample. This should allow us to evaluate thoroughly the influence of IAA and ABA in the induction process of the wound-inducible genes.

Objective 3) Do wounding, systemin, or jasmonic acid affect polar auxin transport?

Figure 6 illustrates that several inhibitors of polar auxin transport can block the wound-induction of the *pin*2-CAT gene system. One very attractive hypothesis that would explain how such a wide variety of structurally different biochemicals (Figure 1) could induce a single gene, is that these various inducers of the *pin*2 gene system might affect a single physiological response such as polar auxin transport. For example, each of these compounds could stimulate polar auxin transport, thereby reducing IAA within cells and derepressing the gene by a common signal transduction mechanism. Further, because TIBA also blocks the natural wound-response, it is possible that wounding also stimulates increased auxin transport that results in a reduced level of IAA within those plant tissues that express the *pin*2-CAT gene.

To examine the truth of this hypothesis, we have designed a series of experiments to measure polar auxin transport in both wounded and non-wounded tissues. Also, we will also examine the effects of known signals such as jasmonic acid and systemin on IAA transport. To conduct these

experiments, we will use modifications of the experiments of Jones (1990). However, because we will be measuring IAA transport out of leaves, we will measure the rate and quantity of IAA transport in the petiole of either wounded or unwounded leaves. We realize that taking the petiole samples for assay will be wounding the leaf, however, because it takes several hours for the wound response to occur we expect that the pre-wounded leaves will show a different pattern from the unwounded leaves. If however, the assay of Jones proves recalcitrant to measuring a difference, then we will try to measure auxin transport by an alternative method. The method of Parker and Briggs (1990) is more rapid than the method of Jones.

If we observe that wounded leaves show an increase in polar auxin transport, then we will extend these studies to examine leaves from plants treated with the various *pin*2 inducers, especially jasmonic acid and systemin.

Assays of IAA Transport

Leaves from an inbred line of our transgenic tobacco plants will be wounded or left unwounded. The leaves will be removed with the petiole still attached to the plant. We will carefully remove leaf blade by slicing with a single stroke of a clean razor blade. This method has causes minimal induction of the wound-inducible gene system (M.K. Walker-Simmons, personal communication). We will use the petioles from both the wounded and unwounded leavesfor assaying auxin transport.

Method of Jones -- The petioles (3 to 4 cm long) from both wounded and unwounded will be removed from the plant and inserted into a small vial containing a Murashige-Skoog (MS) liquid medium containing radiolabeled IAA. Petioles will be placed in the radioactive IAA solution in both the upright and the inverted position. After a 30 minute incubation period, the petioles will be removed, washed 3x with 95% ethanol, and 1% Triton X 100. The part of the petiole that was in contact with the radioactive solution will be discarded, and the remaining part of the petiole stood into a Murashige-Skoog agar medium. Then, small agar blocks will be placed on the upper end of the petiole. Every 20 minutes, the agar blocks will be replaced with new blocks, and the blocks will be extracted into scintillation fluid and counted. Samples from 15 to 20

petioles will be averaged for both the wounded and unwounded leaves to determine whether wounding causes an increase in either the rate or the amount of transported IAA.

Method of Parker and Briggs -- In this method the petioles will remain on the plant. Radiolabeled IAA will be applied in small amounts to the tip of the petiole, and the petioles incubated at constant temperature. Upon application of the drop, and at 15 min intervals thereafter for 60 minutes, the petioles will be wiped upwards several times, gently, with a Kimwipe soaked in 95% ethanol, and 1% Triton X-100. The petioles will then be cut into 1 mm pieces, and the radioactivity is extracted overnight into Aqueous Counting Scintillant in the dark and counted the next day. Samples from 15 to 20 petioles at each 15 minute timepoint will be averaged to determine the levels of IAA transported through the petioles.

We will also compare directly the levels of IAA transported in both unwounded and wounded leaves. We will apply [^{14}C]-IAA to the tip of leaves, either by injecting small volumes or by using ethanol (Parker and Briggs, 1990) to label the endogenous pool of IAA. After various periods of incubation, we will divide the leaf blade or the midvein into small (3 to 5 mm) segments, and process the samples as described above. If we observe a difference between the unwounded and wounded leaves, we will assess auxin transport in leaves treated with the inducers listed in Figure 1, especially systemin and jasmonic acid. In addition we will also evaluate the anti-auxins PCIB and 2,5,6 triiodobenzoic acid which function to

Objective 4) <u>Examine the expression of the *pin*2-CAT gene in plants having elevated or reduced levels of IAA.</u>

To further establish the role of IAA in the induction process for the *pin*2-CAT gene in our transgenic tobacco, we will combine transgenes expressing the *iaa*M with the *pin*2-CAT gene and the *iaa*L with the *pin*2-CAT gene. Transgenic plants expressing high levels of IAA have previously been prepared by Dr. Harry Klee of the Monsanto Co., Saint Louis, Mo. These plants contain the *iaa*M gene from *Pseudomonas syringae*, subspecies *savastanoi* under the control of the CaMV ^{19}S promoter. The *iaa*M gene encodes tryptophan monooxygenase that synthesizes IAA increasing IAA in their foliage by almost 10 fold. In addition, Dr. Klee has also prepared transgenic plants containing the *iaa*L

gene also from *P. savastanoi* (Romano, et al., 1991). The protein encoded by the *iaa*L gene, indoleacetic acid (IAA)-lysine synthetase catalyzes the conjugation of IAA with lysine to form N^{ϵ}-indole-3-acetyl-L-lysine. This reduces IAA within the plant cells by up to 19 fold (Klee, et al., 1987; Romano, et al., 1991). Both are dominant markers, and a single copy of the gene is responsible for changing auxin within the plant cells. Both transgenic plants are available to us (see Appendix 8).

Each the genes used in this portion of this work (^{19}S-*iaa*M, ^{35}S-*iaa*L, and *pin*2-CAT) are dominant and the plants available to us are homozygous for these genes. Therefore, by simply crossing our Tr25 plants (homozygous for *pin*2-CAT) with either the ^{19}S-*iaa*M or the ^{35}S-*iaa*L plants (both homozygous for their genes) all the progeny of these crosses should be of the correct genotype to evaluate the role of IAA on the induction of the *pin*2-CAT gene.

The progeny of these crosses will be analyzed for the presence of the correct transgenes by PCR, using gene specific oligonucleotides. We will also determine the levels of IAA within their leaves (see objective 3 above). Those plants whose levels of IAA are dramatically elevated or reduced we will choose for further analysis. First, we will examine the wound-induction of the *pin*2-CAT gene in these plants with altered IAA levels.

If the hypothesis that IAA regulates the *pin*2 induction system is correct, we expect to find that the *iaa*M gene that elevates IAA levels in plant tissues will inhibit the wound-induction of the *pin*2-CAT gene. We further expect that the *iaa*L gene which lowers IAA in plant tissues should facilitate the wound-induction of the *pin*2-CAT gene. If we can lower IAA enough, this may even make the *pin*2-CAT gene to become constitutively expressed. Thus, these experiments represent a direct test of our hypothesis on the involvement of IAA in the wound-induction process. We will also test those compounds shown in Figure 1 for their ability to induce the *pin*2-CAT gene system in these plants having altered auxin levels.

Implications of the Work

The proteinase inhibitor gene system in solanaceous plants is one of the best characterized inducible gene systems in plants. Yet, there is much that is not known about the regulation of the pathways that induce these genes following wounding. In this proposal, we are asking some very basic questions about the mechanisms of induction of these genes. First, we know there are many very different biochemicals that can induce the Inhibitor II gene system. Yet, we do not know how these compounds interact. Our first goal is to determine if these inducers share a single or multiple signal transduction pathways. Further, we will determine whether auxin is a common inhibitor of these inducers. This should provide new knowledge on signal transduction pathways affecting the *pin2 gene* system in plants. Second, we have preliminary evidence that polar auxin transport may play a role in the activation of the wound-inducible genes. We will carefully examine this question, because *this hypothesis provides a single cohesive framework for the induction of the pin2 genes by all the known inducers of the system*. Further, because the *pin2* gene system is inducible not only in the solanaceae, but also in woody plants (Klopfenstein, et al., 1991), and recent collaborative work with Ray Wu indicates that this system is also inducible in the monocot rice (unpublished), elucidating the underlying mechanisms of induction of this wound-inducible gene system should lead to basic advances in our understanding of plant defenses in general.

LITERATURE CITED

Andreae, W.A. and Good, N.E. (1955) The formation of indoleacetyl-aspartic acid in pea seedlings. Plant Physiol. 30:380-382.

Avery, G.S. (1935) Differential distribution of a phytohormone in the developing leaf of *Nicotiana*, and its relation to polarized growth. Torrey Bot. Club Bull. 62:313-330.

Bevan, M. and Northcote, D.H. (1979) The interaction of auxin and cytokinin in the induction of phenylalanine ammonia-lyase in suspension cultures of *Phaseolus vulgaris*. Planta. 147:77-81.

Bishop, P.D., Makus, D., J. Pearce, G. and Ryan, C. A. (1981) Proteinase inhibitor-inducing factor activity in tomato leaves resides in oligosaccharides enzymatically released from cell walls. Proc. Natl. Acad. Sci. USA. 78:3536-3540.

Bishop, P., Pearce, G., Bryant, J. and Ryan, C. (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves: Identity and activity of poly- and oligogalacturonide fragments. J. Biol. Chem. 259:13172-13177.

Bower, P.J., Brown, H., M. and Pruves, W.K. (1976) Auxin biogenesis: Subcellular compartmentation of indoleacetaldehyde reductases in cucumber seedlings. Plant Physiol. 57:850-854.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72:248-254.

Chang, Y.-P. and Jacobs, W.P. (1973) The regulation of abscission and IAA by senescence factor and abscisic acid. Amer. J. Bot. 60:10-16.

Cleveland, T., Thornburg, R. and Ryan, C. (1987) Molecular Characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol. Biol. 8:199-207.

Cohen, J.D., Baldi, B.G. and Pernise, S.J. (1986) $^{13}C_6$-[Benzene Ring]-Indole-3-acetic acid: A new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol. 80:14-19.

Dixon, R.A. (1986) The phytoalexin response: Elicitation, signalling and control of host gene expression. Biol. Rev. 61:239-291.

Doherty, H.M., Selvendran, R.R. and Bowles, D.J. (1988) Physiol. Mol. Plant Pathol. 33:377-384.

Farmer, E., E. and Ryan, C.A. (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA. 87:7713-7716.

Fox, E.A. (1986) Isolation and characterization of a proteinase inhibitor II gene from *Lycopersicon esculentum*. PhD Dissertation. Washington State University, Pullman, WA.

Gibson, R.A., Barrett, G. and Wightman, F. (1972) Biosynthesis and metabolism of indol-3-yl-acetic acid: III. Partial purification and properties of a tryptamine-forming L-tryptophan decarboxylase from tomato shoots. J. Exp. Bot. 23:775-786.

Goodwin, T.W. and Mercer, E.I. (1983) Introduction to Plant Biochemistry. Pergamon Press. New York. p 677

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2:1044-1051.

Graham, J., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C. (1985a) Wound-induced proteinase inhibitors from tomato-leaves. I. The cDNA-deduced primary structure of pre-inhibitor I. J. Biol. Chem. 260:6555-6560.

Graham, J., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C. (1985b) Wound-induced proteinase inhibitors from tomato-leaves. II. The cDNA-deduced primary structure of pre-inhibitor II. J. Biol. Chem. 260:6561-6564.

Graham, J.S., Hall, G., Pearce, G. and Ryan, C.A. (1986) Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta. 169:399-405.

Green, T. and Ryan, C. (1972) Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. Science. 175:776-777.

Green, T.R. and Ryan, C.A. (1973) Wound-induced proteinase inhibitor in tomato leaves: Some effects of light and temperature on the wound response. Plant Physiol. 51:19-21.

Henderson, J.H.M. and Patel, C.S. (1972) Oxindole-3-acetic acid: Physical properties and lack of influence on growth. Plant Physiol. 27:441-442.

Johnson, R. and Ryan, C.A. (1990) Wound-inducible potato inhibitor II genes: Enhancement of expression by sucrose. Plant Mol. Biol. 14: 527-536.

Jones, A.M. (1990) Location of transported auxin in etiolated maize shoots using 5-azidoindole-3-acetic acid. Plant Physiol. 91:1154-1161.

Katekar, G.F. and Geissler, A.E. (1975) Auxin transport inhibitors: Fluorescein and related compounds. Plant Physiol. 56:645-646.

Katekar, G.F. and Geissler, A.E. (1977a) Auxin transport inhibitors. II. 2-(1-Pyrenoyl)benzoic acid, a potent inhibitor of polar auxin transport. Aust. J. Pl. Physiol. 4:321-325.

Katekar, G.F. and Geissler, A.E. (1977b) Auxin transport inhibitors: III. Chemical requirements of a class of auxin transport inhibitors. Plant Physiol. 60:826-829.

Keil, M., Sanchez-Serrano, J., Schell, J. and Willmitzer, L. (1986) Primary structure of a proteinase inhibitor II gene from potato (*Solanum tuberosum*). Nucl. Acids Res. 14:5641-5650.

Keil, M., Sanchez-Serrano, J. and Willmitzer, L. (1989) Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. EMBO J. 8:1323-1330.

Kernan, A. and Thornburg, R.W. (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II--chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. 91:73-78.

Klee, H.J., Horsch, R.B., Hinchee, M.A., Hein, M.B., Hoffmann, N.L. (1987) The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes Dev. 1:86-96.

Klopfenstein, N.B., Shi, N.-Q., Hall, R.B., McNabb, S.E. and Thornburg, R.W. (1991) A wound-inducible promoter from an herbaceous dicot functions correctly in the woody perennials: Potato proteinase inhibitor II in poplar trees. Can. J. For. Res. 21:1321-1328.

Labarca, C., Nicholls, P.B. and Bandurski, R.S. (1965) A partial characterization of indole acetylinositol from *Zea mays*. Biochem Biophys Res Commun. 20:641-644.

Lawton, S., Dixon, R., Hahlbrock, H. and Lamb, C. (1983) Elicitor induction of mRNA activity: Rapid effects of elicitor on phenylalanine ammonia-lyase and chalcone synthase mRNA activities in bean cells. J. Biochem. 130:131-139.

Lee, J., Brown, W., Graham, J., Pearce, G., Fox, E., Dreher, T., Ahern, K., Pearson, G. and Ryan, C. (1986) Molecular characterization and phylogenetic studies of a wound-inducible proteinase inhibitor I gene in *Lycopersicon* species. Proc. Natl. Acad. Sci. USA. 83:7277-7281.

Li, X., Lamotte, C.E., Stweart, C. Zhang, S., Cloud, N.P., and Ware, J. (accepted for publication) A new method for the quantitation of IAA and ABA in a single plant sample. J. Plant Growth. Regul.

Liu, S.-T., Gruenert, D. and Knight, C.A. (1978) Bound form indole-3-acetic acid synthesis in tumorous and non-tumorous species of *Nicotiana*. Plant Physiol. 61:50-53.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15:473-497.

Nonhebel, H.M. (1982) PhD Dissertation Thesis. University of Glasgow.

Olney, H.O. (1968) Growth substances from *Neratrum tenuipetalum*. Plant Physiol. 43:292-302.

Parker, K.E. and Briggs, W.R. (1990) Transport of indoleacetic acid in intact corn coleoptiles. Plant Physiol. 94:417-423.

Pearce, G., Strydon, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science. 253:895-898.

Peña-Cortez, H., Sanchez-Serrano, J.J., Mertens, R., Willmitzer, L. and Prat, S. (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc. Natl. Acad. Sci. USA. 86:9851-9855.

Pengelly, W.L., Bandurski, R.S. and Schulze, A. (1981) Validation of a radioimmuno assay for Indole-3-acetic acid using gas chromatography-selected ion monitoring- mass spectrometry. Plant Physiol. 68:96-98.

Phelps, R.H. and Sequeira, L. (1967) Synthesis of Indoleacetic acid via tryptamine by a cell-free system from tobacco terminal buds. Plant Physiol. 42:1161-1163.

Piskornik, Z. and Bandurski, R.S. (1972) Purification and partial characterization of a glucan containing indole-3-acetic acid. Plant Physiol. 50:176-182.

Romano, C., Hein, M., and Klee, H. (1991) Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of *Pseudomonas*. Genes Dev. 5:438-446.

Ryan, C.A. (1966) Chymotrypsin inhibitor I from potatoes: Reactivity with mammalian, plant, bacterial and fungal proteinases. Biochem. 5:1592-1596.

Ryan, C.A. (1968) Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. Plant Physiol. 43:1859-1865.

Ryan, C.A. (1978) Proteinase inhibitors in plant leaves: A biochemical model for pest-induced natural plant protection. TIBS. 5:148-150.

Ryan, C.A. (1981) Proteinase Inhibitors. *in* The Biochemistry of Plants: A comprehensive treatise. Vol. 6. *ed.* P. K. Stumpf and E. E. Conn. Academic Press. New York. p 351-370

Sanchez-Serrano, J., Schmidt, R., Schell, J. and Willmitzer, L. (1986) Nucloetide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. Mol. Gen. Genet. 203:15-20.

Schneider, E:A. and Wightman, F. (1978) Auxins. *in* Phytohormones and Related Compounds: A comprehensive treatise. *ed.* Elsevier. Amsterdam. p 29-105

Songstad, D.D., De Luca, V., Brisson, N., Kurz, W.G.W. and Nessler, C.L. (1990) High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol. 94:1410-1413.

Thompson, K.-S., Hertel, R., Muller, S. and Tavares, J.E. (1973) 1-N-Napthylphthalamic acid and 2,3,5-triiodobenzoic acid: *In vitro* binding to particulate cell fractions and action on auxin transport in corn coleoptiles. Planta. 109:337-352.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. and Ryan, C.A. (1987a) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA. 84:744-748.

Thornburg, An, G., Cleveland, T.E. and Ryan., C.A. (1987b) Characterization and expression of a wound-inducible proteinase inhibitor II gene from potato. Basic Life Sci. 41:121-129.

Thornburg, R.W., Kernan, A. and Molin, L. (1990) CAT protein is expressed in transgenic tobacco in field tests following attack by insects. Plant Physiol. 92:500-505.

Thornburg, R.W. (1991) Modes of expression of a wound-inducible gene in field trials of transgenic plants. *in* Biotechnology Field Test Results. *ed.* D. R. MacKenzie. Washington DC. (in press)

Thornburg, R.W. and Li, X. (1991) Wounding of the foliage of *Nicotiana tabacum* causes a decline in the levels of endogenous foliar IAA. Plant Physiol. 96:802-805.

Walker-Simmons, M. and Ryan, C.A. (1984) Proteinase inhibitor synthesis in tomato leaves: Induction by chitosan oligomers and chemically modified chitosan and chitin. Plant Physiol. 76:787-790.

Weiss, J.S. (1966) Effect of oxindoles on the growth of tobacco tissue cultures. Nature. 211:1216-1217.

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Publications

1. Molecular characterization of a wound-inducible inhibitor I gene from Potato and the processing of its mRNA and protein. T.E. Cleveland, R.W. Thornburg, and C.A. Ryan. Plant Mol. Biol. , 8:199-207 (1987).

2. Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R., and Ryan, C.A. Proc. Natl. Acad. Sci USA 84:744-748 (1987).

3. Characterization and expression of a wound-inducible proteinase inhibitor II gene from potato. R.W. Thornburg, G. An, T.E. Cleveland, and C.A. Ryan. *in* Tailoring Genes for Crop Improvement: An agricultural perspective. *eds.* Bruening, G., Harada, J., Kosuge, T., and Hollaender, A. (1987) Basic Life Sciences, 41:121-130.

4. A possible role for 3' sequences of the wound-inducible potato proteinase inhibitor II K gene in regulating gene expression. G. An, R.W. Thornburg, R. Johnson, G. Hall, and C.A. Ryan. *in* NATO ASI Series A: Life Sciences. 140:377-384. *eds* von Wettstein, D. and Chua, N.-H. New York, Plenum Press

5. Regulation of expression of proteinase inhibitor genes in food crops. C.A. Ryan, G. Pearce, G. An, and R.W. Thornburg, Acta Horticulturae, 207:31-39 (1987).

6. An, G., Mitra, A. Choi, H.K., Costa, M.A. An, K., Thornburg, R.W., and Ryan, C.A. (1989) Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1:115-122.

7. Kernan, A. and Thornburg, R.W. (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II--chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol. 91:73-77.

8. Thornburg, R.W., Kernan, A. and Molin, L. (1990) CAT protein is expressed in transgenic tobacco in field tests following attack by insects. Plant Physiol. 92:500-505.

9. C.A. Ryan, T. Moloshok, G. Pearce, G. An, R.W. Thornburg, G. Hall, R. Johnson, E.E. Farmer, and C. Palm. (1990) Engineering proteinase inhibitor genes for plant defense against predators. Journal of the Iowa Academy of Science 97:9-14.

10. Negrerie, M., Bellefeuille, S.M., Whitham, S., Petrich, J.W., and Thornburg, R.W. (1990). A novel noninvasive in-situ probe of protein structure and dynamics. J. Am. Chem. Soc. 112:7419-7421.

11. Thornburg, R.W. (1990) New approaches to pest resistance in trees. AgBiotech News and Information. 2:845-849.

12. Huang, J.-T., Hack, E. Thornburg, R.W., and Myers, A.M. (1990). A yeast mitochondrial leader peptide functions *in vivo* as a dual-targeting signal for both mitochondria and chloroplasts. Plant Cell 2:1249-1260.

13. Thornburg, R.W. Modes of Expression of a Wound-inducible Gene in Field Trials of Transgenic Plants. (1991) *in* Biotechnology Field Test Results, ed. MacKenzie, D.R. and Henry, S.C. Agricultural Research Institute, Bethesda, MD. pp. 147-154.

14. Klopfenstein, N.B., Shi, N.-Q., Hall, R.B., McNabb, S.E. and Thornburg, R.W. (1991) A wound-inducible promoter from a herbaceous dicot functions correctly in the woody perennials: Potato proteinase inhibitor II in poplar trees. Can. J. For. Res. 21:1321-1328.

15. Thornburg, R.W., and Li, X. (1991) Auxin levels in decline following wounding of tobacco leaves. Plant Physiol. 96:802-805.

16. Thornburg, R.W., Park, S., and Li, X. Hormonal Control of Proteinase Inhibitor II Genes in Transgenic Tobacco. (1992, in press; Molecular Mechanisms of Plant Gene Expression ed. Verma, D.P.).

17. Santoso, D. and Thornburg, R.W. (accepted. 1992) Isolation and characterization of UMP synthase mutants from haploid cell suspensions of *Nicotiana tabacum*. Plant Physiol.

18. Park, S. and Thornburg, R.W., Cloning and characterization of a proteinase inhibitor II gene that is not wound-inducible. (submitted).

19. Negrérie, M., Whitham, S., Petrich, J.W., and Thornburg, R.W. Incorporation of the photophysical probe, 7-azatryptophan into bacterial protein. (submitted).

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Dr. J. W. Baynes, Department of Chemistry, University of South Carolina
Dr. J. Baseman, Department of Microbiology, University of Texas HSC/ San Antonio
Dr. C. A. Ryan, Institute of Biological Chemistry, Washington State University

BIOGRAPHICAL SKETCH

Sanggyu Park
Graduate Student
Department of Biochemistry and Biophysics
B406 Agronomy Hall
Iowa State University
Ames Iowa 50011

REDACTED

Education

Seoul National University, Seoul, Korea, BS, Agricultural Chemistry, 1982
Seoul National University, Seoul, Korea, MS, Agricultural Chemistry, 1984
Iowa State University, Ames, Iowa, PhD, Biochemistry and Biophysics, 1991

Employment Experience

1982 - 1986 Graduate student, Department of Agricultural Chemistry, Seoul National University, Korea
1986 - 1991 Graduate Student, Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa , PhD, 1991
1991 - pres Post-Doctoral Fellow with Dr. Robert Thornburg, Department of Biochemistry and Biophysics, Iowa State University, Ames

Publications

1) Jin Jung, Yang-Kee Kim and Sanggyu Park (1983) Relationship between fatty acid composition of phospholipid from leaves and cold tolerance of rice plants J. Kor. Agric. Chem. Soc. 26: 58-64

2) Jin Jung and Sanggyu Park (1984) Biochemical study on mechanism of chilling injury in rice. Seoul Natl Univ. Col. of Agric. Res.9: 75-82.

3) Jin Jung, Sanggyu Park, Sang-Kee Lee and Se-Ho Kim (1985) Determination of Respiratory activity of mitochondria and submitochondrial particles using dropping mercury electrode. J. Kor. Agric Chem. Soc. 28:271-277.

4) Thornburg, R.W., Park, S., and Li, X. Hormonal Control of Proteinase Inhibitor II Genes in Transgenic Tobacco. (1991, in press; Molecular Mechanisms of Plant Gene Expression ed. Verma, D.P.).

5) Park, S. and Thornburg, R.W. Cloning and characterization of a proteinase inhibitor II gene which is constitutively expressed. (submitted).

6) Park, S. and Thornburg, R.W. Inhibitors of Auxin Transport Uncouple Wounding from Induction of Proteinase Inhibitor II-CAT Constructs in Transgenic Tobacco. (in preparation).

BIOGRAPHICAL SKETCH

Tad Kornaga
Graduate Student
Department of Biochemistry and Biophysics
B406 Agronomy Hall
Iowa State University
Ames Iowa 50011

REDACTED

Education

Cracow Agricultural University, Cracow, Poland, BS, Agricultural Sciences, 1982

Employment Experience

1987 - 1991 Technician in the laboratory of Dr. Robert W. Thornburg, Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa

1991 - pres Graduate Student, Department of Biochemistry and Biophysics, Iowa State University, Ames

Publications

1) Kornaga, T., and Thornburg, R.W. Reconstruction of a "lost" tobacco genetic instability by an interspecific cross of *Nicotiana langsdorffii* and *N. sanderae*: Analysis indicates the presence of a transposable element. (in preparation).

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

OMB Approved No. 0524-0022
Expires 6/93

BUDGET

Year 1 (07/01/92 til 06/30/93)

Organization and Address

Iowa State University
Ames, Iowa 50011

USDA Grant No.

Principal Investigator(s)/Project Director(s)

Robert Thornburg

		CSRS FUNDED WORK MONTHS			Duration Proposed Months: 12 ISU COST SHARED	Duration Proposed Months: 12 FUNDS REQUESTED BY PROPOSER	Duration Proposed Months: 12 FUNDS APPROVED BY CSRS (If different)
A.	Salaries and Wages	Calendar	Academic	Summer			
	1. 2 No. of Senior Personnel						
	a. 1 (Co)-PI(s)/PD(s)						
	b. 1 Senior Associates	R			REDACTED		
	2. No. of Other Personnel (Non-Faculty)						
	a. 1 Research Associates/Postdoctorate	R					
	b. Other Professionals						
	c. 1 Graduate Students					REDACTED	
	d. 2 Prebaccalaureate Students						
	e. Secretarial-Clerical						
	f. Technical, Shop and Other						
	Total Salaries and Wages ➤				REDACTED		
B.	Fringe Benefits (If charged as Direct Costs)						
C.	**Total Salaries, Wages, and Fringe Benefits** (A plus B) ➤						
D.	Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					14,000	
E.	Materials and Supplies					12,000	
F.	Travel						
	1. Domestic (Including Canada)					1,000	
	2. Foreign (List destination and amount for each trip)						
G.	Publication Costs/Page Charges					1,000	
H.	Computer (ADPE) Costs						
I.	All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)					18,480	
J.	**Total Direct Costs** (C through I) ➤						
K.	**Indirect Costs** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 42% (MTDC) = (J - D) * 0.42				REDACTED		
L.	**Total Direct and Indirect Costs** (J plus K) ➤						
M.	**Other** ➤						
N.	**Total Amount of this Request** ➤				REDACTED		$
O.	**Cost Sharing**						

NOTE: Signatures required only for Revised Budget — *This is Revision no.* ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director Robert Thornburg		
Authorized Organizational Representative Richard E. Hasbrook	Richard E. Hasbrook	1-30-92

Form CSRS-55 (9/89)

2023129019

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

OMB Approved 0524-0022
Expires 3/92

BUDGET

Year 2 (07/01/93 til 06/30/94)

Organization and Address

Iowa State University
Ames, Iowa 50011

USDA Grant No.

Principal Investigator(s)/Project Director(s)

Robert Thornburg

	CSRS FUNDED WORK MONTHS			Duration Proposed Months: 12 ISU COST SHARED	Duration Proposed Months: 12 FUNDS REQUESTED BY PROPOSER	Duration Proposed Months: 12 FUNDS APPROVED BY CSRS (If different)
A. Salaries and Wages	Calendar	Academic	Summer			
1. 2 No. of Senior Personnel						
a. 1 (Co)-PI(s)/PD(s)	25%				REDACTED	
b. 1 Senior Associates	25%					
2. ____ No. of Other Personnel (Non-Faculty)						
a. 1 Research Associates/Postdoctorate	100%					
b. ____ Other Professionals						
c. 1 Graduate Students					REDACTED	
d. 2 Prebaccalaureate Students						
e. ____ Secretarial-Clerical						
f. ____ Technical, Shop and Other						
Total Salaries and Wages ➤						
B. Fringe Benefits (If charged as Direct Costs)					REDACTED	
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ➤						
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.					1,400	
E. Materials and Supplies					12,000	
F. Travel						
1. Domestic (Including Canada)					1000	
2. Foreign (List destination and amount for each trip)						
G. Publication Costs/Page Charges					1000	
H. Computer (ADPE) Costs						
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)					18,480	
J. Total Direct Costs (C through I) ➤						
K. Indirect Costs (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 44% (MTDC) = (J - D) * 0.44					REDACTED	
L. Total Direct and Indirect Costs (J plus K) ➤					REDACTED	
M. Other ➤						
N. Total Amount of this Request ➤						$
O. Cost Sharing						

NOTE: Signatures required only for Revised Budget — This is Revision no. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director Robert Thornburg		
Authorized Organizational Representative Richard E. Hasbrook	Richard E Hasbrook	1-30-92

Form CSRS-55 (9/89)

2023129020

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

OMB Approved 0524-0022
Expires 8/92

BUDGET

Total Budget (07/01/92 til 06/30/94)

Organization and Address

Iowa State University
Ames, Iowa 50011

USDA Grant No.

Principal Investigator(s)/Project Director(s)

Robert Thornburg

	CSRS FUNDED WORK MONTHS			Duration Proposed Months: 12 ISU COST SHARED	Duration Proposed Months: 12 FUNDS REQUESTED BY PROPOSER	Duration Proposed Months: 12 FUNDS APPROVED BY CSRS (If different)
A. Salaries and Wages	Calendar	Academic	Summer			
1. 2 No. of Senior Personnel						
a. 1 (Co)-PI(s)/PD(s)	25%			$ REDACTED		$
b. 1 Senior Associates	25%					
2. ____ No. of Other Personnel (Non-Faculty)						
a. 1 Research Associates/Postdoctorate	100%					
b. ____ Other Professionals						
c. 1 Graduate Students				REDACTED		
d. 2 Prebaccalaureate Students						
e. ____ Secretarial-Clerical						
f. ____ Technical, Shop and Other						
Total Salaries and Wages ➤						
B. Fringe Benefits (If charged as Direct Costs)				REDACTED		
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ➤						
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					15,400	
E. Materials and Supplies					24,000	
F. Travel: 1. Domestic (Including Canada)					2,000	
2. Foreign (List destination and amount for each trip)						
G. Publication Costs/Page Charges					2,000	
H. Computer (ADPE) Costs						
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.)						
J. Total Direct Costs (C through I) ➤						
K. Indirect Costs (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 42% (MTDC) = (J - D) * 0.42 (0.44 in Year 2)				REDACTED		
L. Total Direct and Indirect Costs (J plus K) ➤						
M. Other ➤				REDACTED		
N. Total Amount of this Request ➤						$
O. Cost Sharing						

NOTE: Signatures required only for Revised Budget — This is Revision no. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director Robert Thornburg		
Authorized Organizational Representative Richard E. Hasbrook	Richard E. Hasbrook	1-30-92

Form CSRS-55 (9/89)

2023129021

Budget Justification

Personnel

ISU will contribute 25% of PI Robert Thornburg's annual salary and associated benefits and indirect costs:

	Year 1	Year 2	Year 3	Total
Thornburg	R			
Benefits,	R	REDACTED		REDACTED
Indirect Costs,	R			
TOTAL				

For the Postdoctoral Fellow a salary increment R is included in years 2 and 3. The graduate student will receive a salary increment R per year in years 2 and 3.

University benefits rates are used: R for the PI, R for the Postdoctoral Fellow. The Graduate Student benefits are R per year.

We are requesting salary support for the following individuals --

1 postdoctoral fellow, Dr. Sanggyu Park, will conduct the polar auxin transport studies, with both unwounded and wounded leaves, and in addition Dr. Park will also conduct the auxin transport studies on the induced leaves.

1 graduate student, Mr. Tadeusz Kornaga, will continue the comparison of the inducing compounds with IAA. Mr. Kornaga has done some of the early work on the comparison of ABA and IAA. Mr. Kornaga will also prepare the samples for IAA analysis that will be done at the Iowa State University Plant Hormone Analysis Facility.

1 graduate student, yet unnamed, will make the crosses between the *iaa* plants and the *pin*2-CAT plants, and analyze their progeny.

Since undergraduate education is a major pursuit of Iowa State University, we are requesting funds to support two part time undergraduate student in the laboratory during the school year. All previous undergraduates who have worked for the PI have been accepted into graduate or professional schools.

Equipment

Image processing apparatus	$14,082
Microcentrifuge	$1,400

In year 1 we are requesting funds for an image processing apparatus. We run so many CAT assays (several thousands each year), that cutting and counting of each sample becomes a rate limiting step due to time on the scintillation counter and the time required to process each TLC plate. We anticipate that this image processing apparatus will save us a significant amount of time for processing our CAT assays. This apparatus can also be used for processing information from the blotting experiments outlined in Objective 3.

In year 2 we are requesting funds to purchase a microcentrifuge. The current microfuge in our laboratory receives heavy use, and frequently breaks. We anticipate that we can keep it running for one additional year.

Other:

Greenhouse -- Greenhouse space for the growth of our plants is approximately $1000 annually.

Ultracentrifuge Service Contract -- We are requesting funds to cover the service contract on one of the Ultracentrifuges located in my laboratory. The cost is $1,880 annually.

Plant Hormone Analyses -- The cost of each hormone analysis is $65 per sample to determine the levels of both IAA and ABA at the Iowa State University Hormone Analysis Facility. For our analysis of IAA in leaves, we anticipate sampling at times (0, 3, 6, 9, 12, 18, 24, and 48 hours after wounding). We propose 5 replicates per sample time, and each replicate will consist of 6 parts (basal, medial and apical -- wounded and non-wounded). Finally the entire experiment will be duplicated. These experiments will be carried out in years 1 and 2. These analyses will be carried out by Dr. Xiaoyue Li, formerly a postdoctoral Fellow with the PI, who currently operates the ISU hormone facility.

	8 sample times
X	6 sample parts
X	5 replicates
X	2 duplication
	X $65
	$31,200

Materials and Supplies

Materials and supplies will include: expendable enzymes and materials used in PCR analysis, radionucleotides for CAT analysis and IAA transport studies, biological media for plant tissue culture.

Travel

I am requesting travel support for the Postdoctoral Fellow and myself to one major national meeting per year. I expect to present the findings of this work at these meetings.

Indirect Costs

Indirect Costs are 42% for 1992-1993 and 44% from then on.

Facilities and Space:

Dr. Thornburg's laboratory is housed in a new wing of the Agronomy Building. This space is occupied by 4 new faculty in various areas of plant molecular biology. Major equipment for this space includes: two L8 ultracentrifuges and rotors (vTi60, SW50.1, Ti70, SW27), three super-speed centrifuges with rotors (SS-34, GSA), A PCR machine, a DNA synthesizer, NewBrunswick incubator shakers, a recording spectrophotometer, a scintillation counter, a dark room, a media prep area, three plant growth rooms and adequate greenhouse space.

Dr. Thornburg has approximately 820 sq.ft. of laboratory space assigned to him in Agronomy Hall, where most of this work will be carried out. This space includes a fume hood for radioisotope work, a microcentrifuge, balances (analytical and preparative), two Nuair sterile hoods for tissue culture, pH meter, shaking incubators, speed vac, cold box, heating blocks, freezers, refrigerators, a -80C freezer, power supplies (low and high voltage), electrophoresis apparati (PAGE, agarose, and DNA sequencing), a transilluminator and a MP-4 camera are also available. All the equipment in this space is new within the last five years. All the major equipment is maintained under service contract by the investigators which are housed in the basement of the agronomy building. A personal computer is available with enough capacity to handle the word processing and DNA analysis requirements of the laboratory is also present.

The PI's laboratory has been approved for use of [H-3], [C-14], [P-32], [S-35] and [I-125] by the Iowa State University Radiation Safety Committee. In addition, the Iowa State University Institutional Biosafety Committee has certified that Dr. Thornburg's laboratory meets the requirements for BL-2 containment. The ISU IBC has also approved studies involving transformation of solanaceous plants (including tobacco, tomato and potato) to antibiotic resistance within the rDNA guidelines of the National Institute of Health. In addition, Dr. Thornburg has requested and been granted permission from the US Department of Agriculture for release to the environment of transgenic plants containing portions of an insect resistance gene. These studies were conducted during the summer of 1988 and 1989 with both tobacco plants and poplar trees. Secretarial services and administrative and purchasing arrangements are available through the Department of Biochemistry and Biophysics.

Iowa State University has recently (in the past 4 years) hired 21 young, new faculty members in various rDNA positions. Iowa State University is currently spending $18 million for biotechnology research equipment. These purchases include: a DNA center, headed by Dr. Robert Benbow, to aid area investigators with DNA sequencing, DNA synthesis, and DNA preparations; a Protein Center, headed by Dr. Louisa Tabatabai, which will maintain a university laboratory for protein analysis and sequencing, and polypeptide synthesis; a Cell Center, headed by Dr. James A. Olson, which houses an EPICS 752 flow cytometer, as well as provides a monoclonal antibody service to area investigators; and a Fermentation Center for the production of large quantities of microorganisms. In addition, construction is underway on a $39.5 million Molecular Biology Building. The PI is scheduled to receive approximately 1200 sq ft of laboratory space in this new building in February of 1992. All the above equipment will move with the PI at that time.

In the near vicinity (6 km) of Iowa State University is the National Animal Disease Center (NADC, a branch of the USDA/APHIS), and the Iowa State Veterinary Medicine Research Institute (VMRI). Both NADC and VMRI have many investigators involved in rDNA work and its application to modern agricultural problems.

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

OMB Approved 0524 0039
Expires 8/92

CURRENT AND PENDING SUPPORTY

Instructions:

1. Record information for active and pending projects. (Concurrent submission of a proposal to other organizations will not prejudice its review by CSRS)
2. All current research to which principal investigator(s) and other senior personnel have committed a portion of their time must be listed, whether or not salary for the person involved is included in the budgets of the various projects.
3. Provide analogous information for all proposed research which is being considered by, or which will be submitted in the near future to, other possible sponsors including other USDA programs.

Name (List PI #1 first)	Supporting Agency and Project Number	Total $ Amount	Effective and Expiration Dates	% of Time Committed	Title of Project
	Current:				
Thornburg, Robert	REDACTED	REDACTED	07/01/91 til 06/30/93	25%	Isolation of Wound-Pathway Mutants in Plants
	Pending:				
Thornburg, Robert	This proposal	REDACTED	07/01/92 til 06/30/95	25%	Auxin Regulation of Proteinase Inhibitor Genes in Transgenic Tobacco
Thornburg, Robert	NSF		07/01/92 til 06/30/96	25%	Auxin Regulation of Proteinase Inhibitor Genes in Transgenic Tobacco
Thornburg, Robert	USDA -- 52.0 / 52.2		07/01/92 til 06/30/95	25%	A Novel Method for Expression of Genes in Plants at Very High Levels

2023129025

R: REDACTED MATERIAL

List of Appendices

Appendix 1

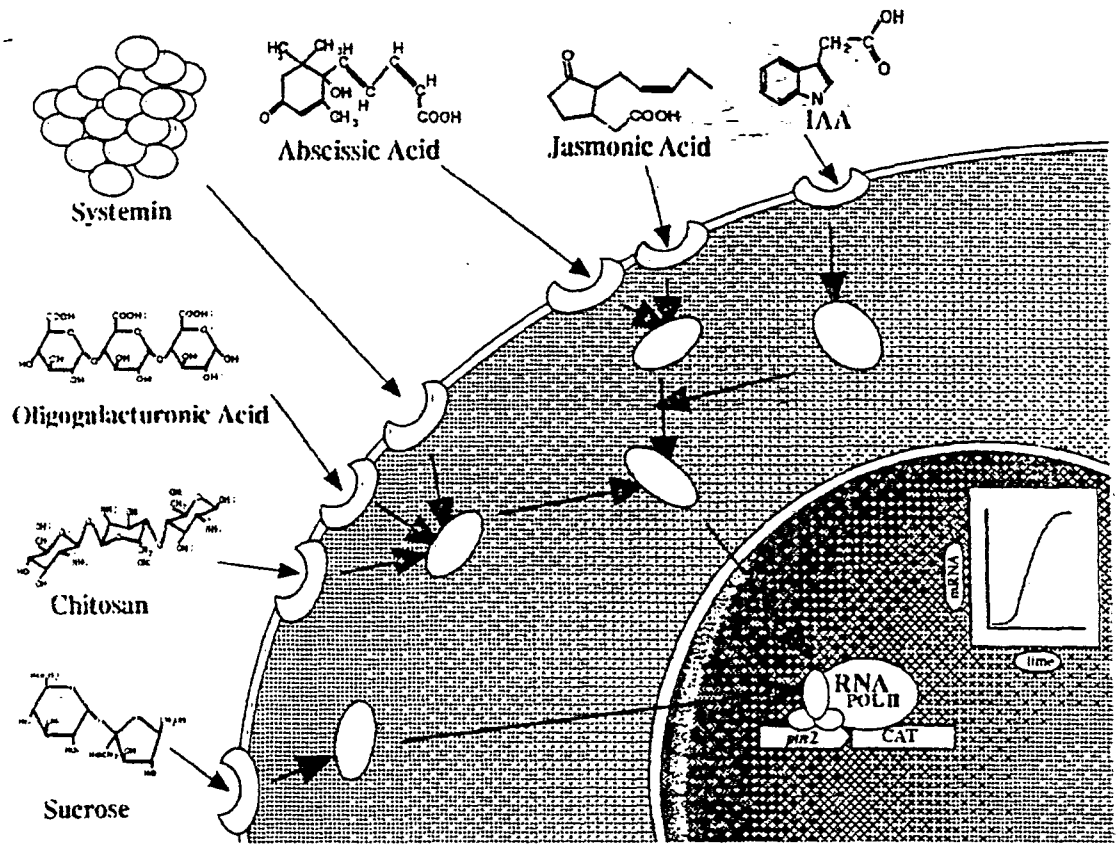


Figure 1) Factors affecting expression of wound-inducible Proteinase Inhibitor genes

There are many biochemical factors that have been found to affect the expression of the wound-inducible proteinase inhibitor genes in solanaceous plants. These biochemical factors include oligo and polysaccharides, (Sucrose, oligogalacturonic acid, and chitosan), as well as plant growth regulators (auxin, abscisic acid, and jasmonic acid). In addition, there has been a recently identified 18 amino acid polypeptide that is termed systemin that induces gene expression at fMolar concentrations. The mechanisms of how these factors affect the expression of the wound-inducible proteinase inhibitor genes is unknown. Nothing is known about signal transduction in this system, nor is there very much known about the transcription initiation complexes that must be formed to obtain gene expression. These are the types of mutants that we expect this scheme would produce. Those that are blocked in the activation of the gene system by cell surface receptor, signal transduction, transcription initiation or possibly others.

Appendix 2

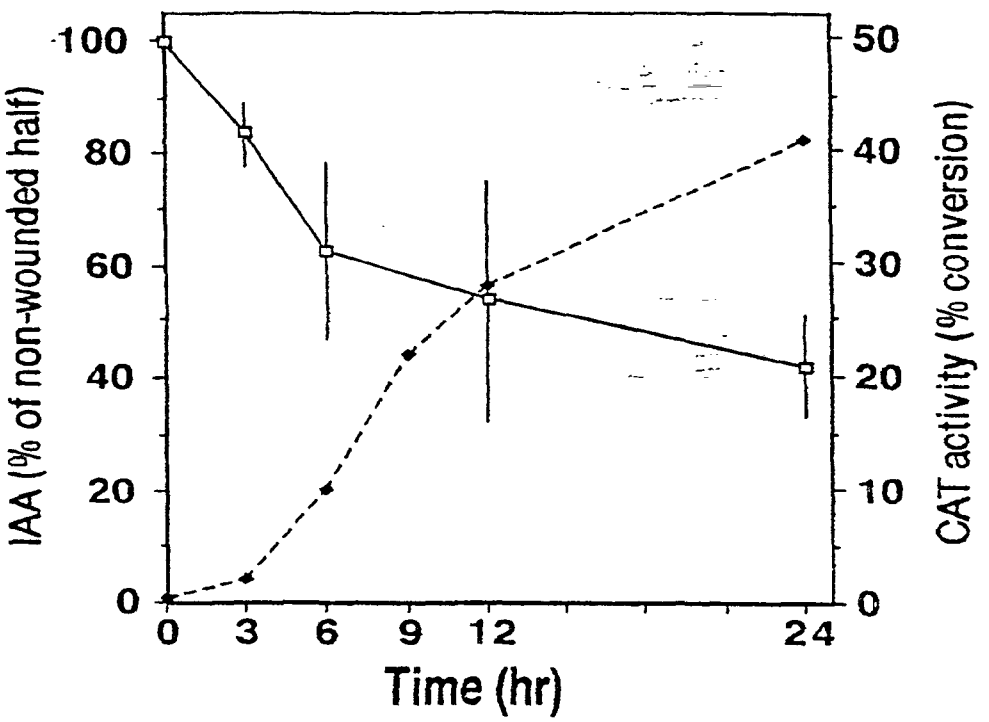


Figure 2) Levels of IAA and CAT activity in transgenic plants following wounding

Plants were wounded by squeezing the leaves between the blades of surgical forceps in a 2 cm wide swath across the whole leaf. After the indicated times, leaves were harvested and examined for CAT activity. Data is expressed as the percentage of conversion of ^{14}C-chloramphenicol into chloramphenicol acetates. In separate experiments, IAA was measured by the half leaf method as described in the text. Data are presented as percentage of IAA present in the wounded leaf half relative to the amount of IAA present in the non wounded leaf half.

Appendix 3

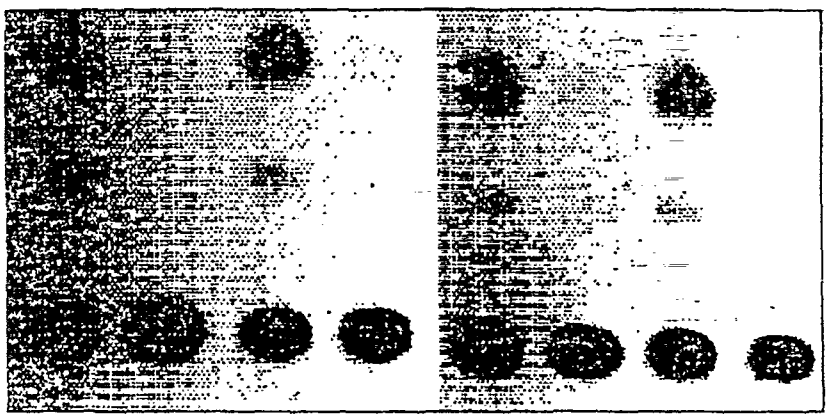
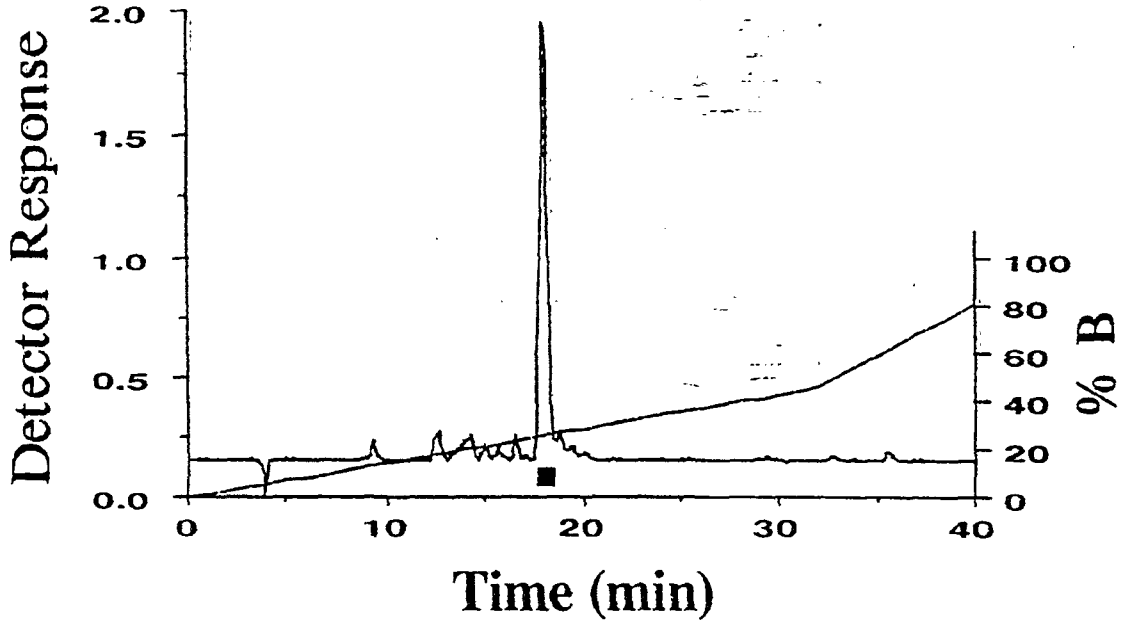


Figure 3) TIBA inhibits wound-induced accumulation of CAT activity
Identical Tr25 plants were sprayed with either water or triiodobenzoic acid. On each plant two consecutive leaves from the central portion of each plant were wounded and one adjacent leaf was left unwounded. After 20 hours these leaves were removed from the plant and assayed for CAT activity. Shown here are the TLC plates showing the levels of CAT activity in both the unwounded and wounded leaves. Lanes 1 to 3 are from a plant sprayed with water. Lanes 4 to 6 are from a plant sprayed with TIBA. Lanes 1 and 4 were unwounded leaves. Lanes 2, 3, 5, and 6 were wounded leaves. In each case 100 ug of total extracted leaf protein was assayed for expression of CAT protein.

Appendix 4



B

Amino Acid	Expected	Determined	Amino Acid	Expected	Determined
Alanine	1	1.02 ± 0.02	Leucine	0	ND
Argenine	1	1.05 ± 0.02	Lysine	3	2.82 ± 0.10
Asparagine	0	ND[a]	Methionine	1	0.88 ± 0.03
Aspartic Acid	2	2.08 ± 0.06	Phenylalanine	0	ND
Cysteine	0	ND	Proline	4	4.04 ± 0.11
Glutamine	0	ND	Serine	2	1.74 ± 0.05
Glutamic Acid	2	2.08 ± 0.05	Threonine	1	0.95 ± 0.02
Glycine	0	ND	Tryptophan	0	ND
Histidine	0	ND	Tyrosine	0	ND
Isoleucine	O	ND	Valine	1	0.98 ± 0.02

[a]ND = not detected

Figure 4) Synthesis and analysis of Systemin

The amino acid sequence of systemin (Pearce, et al., 1991) was used to synthesize an 18 amino acid systemin polypeptide. The peptide was synthesized on an Applied Biosystems Model 340 A peptide synthesizer. After cleavage from the solid phase support, the resulting crude polypeptide was purified by reverse phase HPLC on a C-18 column (Panel A). More than 95% of the eluting material was in the major peak. The purified material in this peak was hydrolyzed and analyzed for amino acids. The table (Panel B) indicates the expected and determined (n = 4) results of this analysis.

Appendix 5

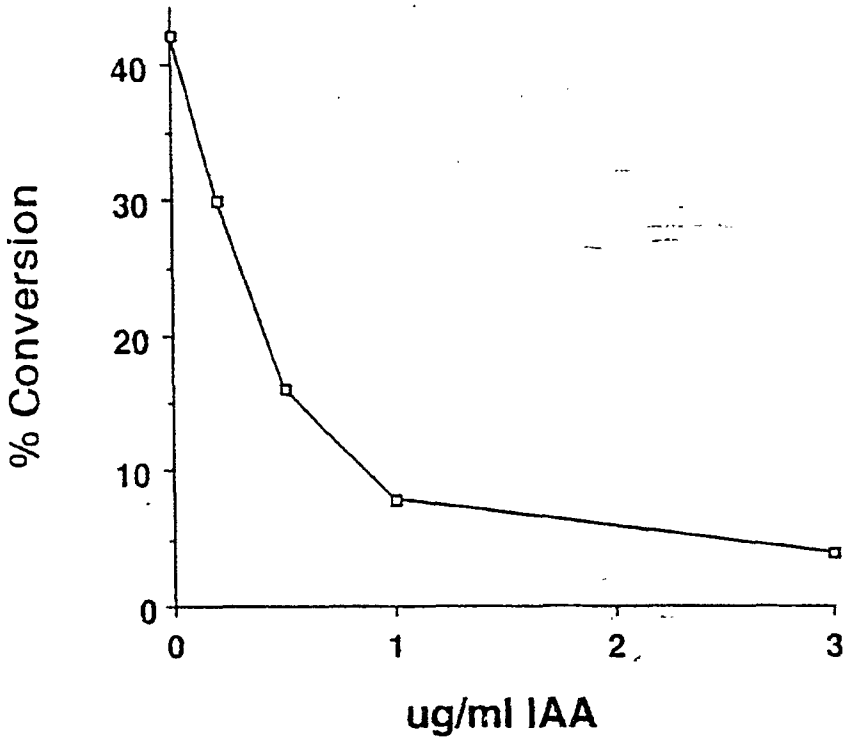


Figure 5) Auxin inhibits ABA induced expression of *pin*2-CAT gene in Tr25 tobacco

Leaves of transgenic Tr25 tobacco plants containing the wound-inducible *pin*2-CAT gene were removed from the plant with a single clean cut of a razor blade and placed in a solution of Murashige-Skoog salts. The leaves were induced to accumulate CAT protein by the addition of ABA. At the same time, varying amounts of IAA were added to the MS liquid salts solution. After 24 hours, the leaves were assayed for CAT activity driven by the Inhibitor II promoter. The data presented show that increasing levels of IAA represses the induction of pin2-CAT by ABA.

Appendix 6

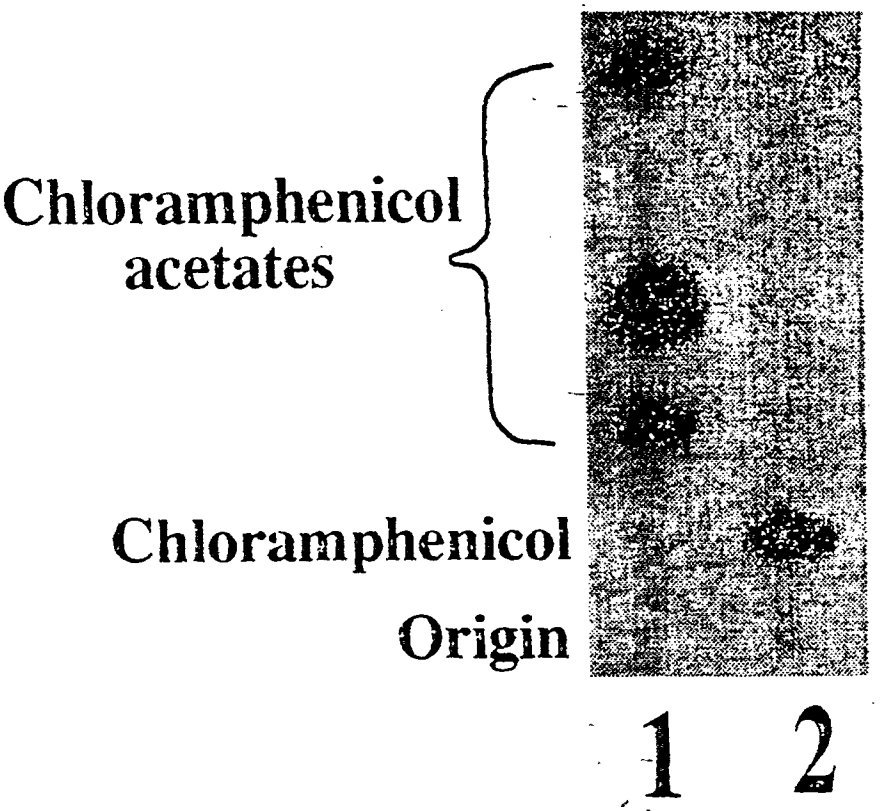


Figure 6) TIBA inhibits ABA induced expression of *pin*2-CAT gene in Tr25 tobacco

Consecutive leaves were removed from transgenic Tr25 tobacco plants with a single clean cut of a razor blade. The petioles of the leaves were then immersed in a solution of Murashige-Skoog salts liquid medium containing 100 ug/ml ABA. The leaf in lanes 1 was sprayed with water, while the leaf in lane 2 was sprayed with TIBA (1 mM). After 18 hours, the leaves were processed for CAT assay, and the assays visualized by thin layer chromatography.

Appendix 7

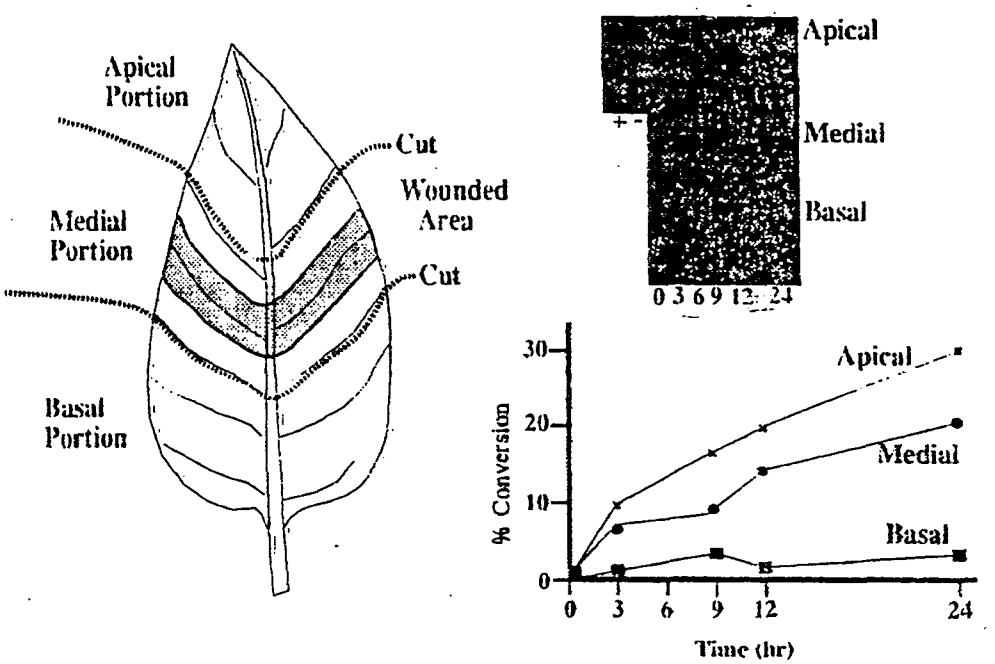


Figure 7) Kinetics of induction of CAT protein in various portions of a wounded leaf.

Leaves of several Tr25 transgenic tobacco plants were wounded as indicated on the leaf diagram (left panel). After an appropriate incubation, leaves were cut into three parts; an apical portion, a medial portion which contained the wounded material and a basal portion. The portions were homogenized and CAT assays were performed. The right hand portion of the figure indicates the accumulation of CAT protein in these portions of the leaf. The insets are the TLC autoradiograms demonstrating the accumulation with time of the CAT protein. Plus (+) and minus (-) in the Apical panel are positive and negative controls containing 10 units of CAT protein ind no added protein respectively. The numbers under the Basal panel represent the time following wounding (hours) until the leaf material was homogenized.

Monsanto APPENDIX 8

Monsanto Company
700 Chesterfield Village Parkway
St. Louis Missouri 63198
Phone (314) 694-1000

11-18-91

Dr. Robert Thornburg
Dept. of Biochemistry and Biophysics
Iowa State University
Ames, Iowa

Dear Dr. Thornburg,

Enclosed please find enclosed pMON518 and pMON690 transgenic tobacco seed. The line designations (19S-iaaM#1, 35S-iaaL#7, etc) correspond to those used in the Genes and Development paper (5:438-446, 1991). I have also enclosed wild type seed from the Samsun line used for transformation as well as an additional pMON518 line (19S-iaaM#2) that has not been described. We are currently quantitating auxin levels in 19S-iaaM#2, which displays much the same phenotype as 19S-iaaM#1.

We would be most interested in any results that you obtain with these plants, especially with respect to levels of free or conjugated IAA. Please feel free to contact either Harry or myself if any questions arise.

Sincerely,

Charley Romano

REDACTED

Plant Physiol. (1989) 91, 73–78
0032-0889/89/91/0073/06/$01.00/0

Received for publication November 17, 1988
and in revised form April 10, 1989

Auxin Levels Regulate the Expression of a Wound-Inducible Proteinase Inhibitor II-Chloramphenicol Acetyl Transferase Gene Fusion *in Vitro* and *in Vivo*[1]

Andrea Kernan and Robert W. Thornburg*

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ABSTRACT

Proteinase inhibitor genes are expressed in solanaceous and leguminous plants following wounding of the foliage by mechanical methods. Previous studies have shown that a cloned proteinase inhibitor II-chloramphenicol acetyl transferase (*pin2*-CAT) chimeric gene is regulated in a wound-inducible manner in transgenic plants. In this study, we analyzed transgenic plant tissues for expression of the *pin2*-CAT gene in response to various plant hormones. We found that CAT activity was induced in tobacco (*Nicotiana tabacum*) callus incubated in the absence of any plant growth regulators. Addition of growth regulators to the medium thus permitted us to measure the effects of these substances on the activity of the *pin2*-CAT gene construction. Cytokinin (BAP) and ethylene (ethophon) even at low concentrations stimulated the expression of CAT activity by 25 to 50%. Abscisic acid at concentrations up to 4.4×10^{-5} molar had no effect upon CAT activity, but increasing auxin (naphthalene acetic acid) levels completely inhibited the synthesis of CAT protein. Gibberellic acid had little effect except at very high concentration (2.9×10^{-5} molar). The kinetics of activation of the *pin2*-CAT gene were quite long (5 to 7 days) when unwounded calli were plated on media lacking auxin. This effect was documented for calli derived from several transformed plants, containing the full, chimeric *pin2*-CAT (pRT45) gene. In addition, calli from tissues transformed with wild-type vectors or from several plants transformed with pRT50 (a noninducible derivative of pRT45) were not induced by plating on media lacking auxin. Other naturally occurring and synthetic auxins had similar effects to naphthalene acetic acid in inhibiting the induction of the chimeric gene fusion. Finally, leaf discs from transformed plants were induced by incubation in MS liquid medium in the presence and absence of naphthalene acetic acid. NAA was also effective in down regulating the chimeric gene in whole plant tissues.

Wounding of plants is responsible for the regulation of a wide variety of gene products in plants (1, 3, 6, 13, 17, 26). The inducible characterized genetic systems include plant defences against microbes and herbivores (13, 17, 19). One of the best characterized examples of the induction of genes following wounding is that of the proteinase inhibitor genes of solanaceous plants (11, 12, 24, 31). In this system, two small gene families (termed proteinase inhibitor I and II), which encode quantitative insect resistance factors, are induced from a quiescent to an active state by wounding.

The complete mechanism by which these genes become induced is not known, but several steps in the process have been elucidated. Wounding of plant leaves releases intravacuolar glycosidases which interact specifically with adjacent cell walls to liberate low mol wt oligosaccharides (2, 6). These low mol wt oligosaccharides have been isolated and can be fed through the petiole of detached leaves to induce the accumulation of the proteinase inhibitors (24). The induction is regulated at the transcriptional level and the levels of mRNA approach 0.7% of total poly(A$^+$) mRNA following multiple wounds (11). The kinetics of induction for both families of proteinase inhibitors are quite long (4–6 h) following wounding before mRNAs begins to accumulate (11). This long preinduction period is unusual in inducible systems indeed even among other wound-inducible systems gene activation is quite rapid (17).

The genes which code for these proteinase inhibitors have been isolated and characterized from both tomato and potato (5, 9, 16–18, 31). The wound-inducible nature of the *pin2*[2] genes was demonstrated independently by two separate groups (23, 31).

This study utilizes whole plants and tissues transformed with the wound-inducible *pin2*-CAT gene to examine the biochemical mechanism of wound-induction of plant defense genes.

MATERIALS AND METHODS

Plant hormones and media components for plant tissue culture were purchased from Gibco Laboratories, Grand Island, NY; Kelco Division of Merck and Co., San Diego; or Sigma Chemical Co., St. Louis. [^{14}C]Chloramphenicol (specific activity = 60 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All other materials were of reagent grade and obtained through local sources.

Plant Lines and Tissue Culture

The construction and description of wound-inducible transformed *Nicotiana tabacum* cv Xanthi plants, was previously

[1] This work was supported by grants from the U.S. Department of Agriculture (87-CRCR-1-2518), the State of Iowa, and the Iowa Biotechnology Council.

[2] Abbreviations: *pin2*, proteinase inhibitor II gene; CAT, chloramphenicol acetyl transferase; NAA, α-naphthalene acetic acid; IAN, indole acetamide; IPA, indole propionic acid; IBA, indole butyric acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; BAP, benzylaminopurine.

described (31). Calli were initiated from these transformed tobacco plants by plating leaf and/or stem pieces onto callus induction medium (Murashige and Skoog (21) [MS] solid agar containing 2 mg/L of NAA, 0.5 mg/L BAP, and 200 mg/L kanamycin sulfate). This procedure produced light yellow, friable, rapidly dividing calli for each of the transformed tobacco lines. Transformed calli were maintained in an illuminated incubator set to 16 h days at 26°C and 8 h nights at 18°C. The calli were expanded on the above medium or a similar medium in which IAA replaced NAA. To induce the activation of the *pin2*-CAT gene in transformed tissues, calli portions (approximately 0.5 cm in diameter) were transferred to basal medium (MS^- agar) containing differing concentrations of phytohormones as indicated. One tobacco cell line, NT-RT45-01, was produced by cocultivation of *N. tabacum* cv Xanthi plants with the wild type *Agrobacterium tumefaciens*, A281. This cell line has been maintained on media without hormones or on regeneration media (MS plus 0.5 BAP) for more than 2 years, yet it has never regenerated plants.

CAT Assays

Extracts of tobacco calli were prepared by grinding the tissue plus an equal volume of homogenization buffer (0.1 M Tris-HCl [pH 8.0], 0.5 M sucrose, 0.1% [w/v] ascorbic acid, 0.1% [w/v] cysteine HCl) in a Con-Torque homogenizer (Eberbach, Corp; Ann Arbor, MI). Following grinding, the expressed juice was twice centrifuged for 5 min at 10,000*g* and the clear supernatant was recovered for protein analysis by the method of Bradford (4). A quantity from each extract containing 100 μg of protein was assayed for CAT activity using [^{14}C]chloramphenicol (100,000 cpm = 1.66 μmol) as substrate (10). Following TLC analysis, the activity of the CAT protein was visualized by exposure to x-ray films. The TLC plates were overlayed onto the exposed film and the radioactive spots corresponding to labeled chloramphenicol acetate and unreacted chloramphenicol were cut out and counted in a Packard liquid scintillation counter. Data are expressed as the percent conversion of chloramphenicol into the acetylated forms of chloramphenicol in 60 min at 37°C.

Plant Leaf Induction

Leaf discs were removed from the leaves of transformed plants by a cork punch and the epidermis peeled from them. These discs were induced by a method of R. Johnson and C. A. Ryan (personal communication). Leaf discs were incubated in Murashige-Skoog liquid medium containing various amounts of naphthalene acetic acid. After 18 to 24 h the leaf disc tissues were ground and CAT assays were run as described above.

RESULTS

Induction of *pin2*-CAT on Basal Medium

To induce the accumulation of CAT protein, calli prepared from plants containing the wound-inducible chimeric *pin2*-CAT gene were placed in tissue culture on basal medium. This caused an activation of the wound-inducible gene and permitted us to begin an investigation of the hormonal effects on the regulation of this gene.

In order to begin these studies on the hormonal regulation of this *pin2*-CAT construction, it first became necessary to determine the kinetics of activation of the chimeric gene in calli transferred to basal medium. Therefore, a callus line from the transformed plant, Tr12 (31), was inoculated for 0 to 10 d on various test media. The amount of CAT activity was measured by TLC assay and was taken to represent the activity of the wound-inducible *pin2* promoter. Figure 1 shows the accumulation with time of CAT activity, expressed as percent conversion of chloramphenicol into chloramphenicol acetates.

The accumulation of CAT activity in the Tr12 calli on basal media was negligible until d 5, then over a 48-h period large amounts of CAT activity were induced. In contrast to this, when calli were plated on the maintenance medium, (basal MS medium plus 2.0 mg/L NAA, 0.5 mg/L BAP) no expression of CAT activity was apparent after 10 d. In addition, when Tr12 calli were plated on basal media for 5 d until CAT activity was just beginning to be expressed, and then they were shifted onto maintenance media, no significant CAT activity was obtained. Thus, shifting onto fresh media containing NAA and BAP reversed this induction process. This implied that the 5-d period of induction may have exhausted some callus component and this depletion leads to activation of the *pin2*-CAT gene.

Auxin Regulates *pin2*-CAT Gene Expression

The two components of the maintenance media that are not found in the basal medium are cytokinin (BAP) and auxin

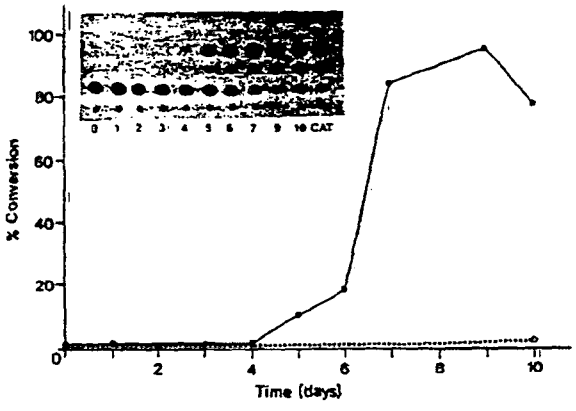

Figure 1. Time course of induction of CAT activity in transgenic Tr12 tobacco calli following transfer to hormone-free medium. The data presented are the percent conversion of chloramphenicol into chloramphenicol acetates. Calli were grown on media containing 2 mg/L NAA, 0.5 mg/L BAP, and on d 0 were transferred onto basal media lacking hormones (closed circles), or complete media containing 2 mg/L NAA, 0.5 mg/L BAP (open circles). In this experiment, three calli at each time point were pooled and homogenized together. The data presented are thus an average of the individual calli. Inset is an autoradiograph showing expression of CAT activity at the indicated days; 0 and CAT are negative and positive CAT enzyme controls.

(NAA). Therefore, the effects of these plant hormones on the regulation of *pin2*-CAT gene expression was investigated (Fig. 2). BAP, when added at even the lowest concentration (8.8×10^{-8} M), stimulated the expression of CAT activity by about 30% over that obtained with the basal medium alone. This stimulation did not significantly change with increasing concentration of BAP up to 4.4×10^{-5} M.

In contrast, when the auxin naphthalene acetic acid was added to the basal medium either alone or in combination with cytokinin, the expression of inducible CAT protein was drastically reduced. Thus, auxin concentrations in the media apparently regulate the *pin2*-CAT gene. The hormone concentration required to give 50% inhibition of expression was 38 μg/L or 2.0×10^{-7} M NAA, which is near to physiological concentrations of IAA in tobacco leaves (28).

When the cytokinin, BAP was added to media containing various levels of NAA, the expression of *pin2*-CAT gene closely resembled the expression found with NAA alone (data not shown). We therefore concluded that auxin levels control expression of CAT activity in these tissues and that the increased activity induced by cytokinins in the complete absence of auxin may not be physiologically relevant.

This phenomenon was initially observed with callus derived from a single transformed plant, Tr12. To determine whether this was a unique phenomenon restricted to this transformant or whether this was a general characteristic of the chimeric wound-inducible *pin2*-CAT gene fusion, calli derived from other transformed plants were tested in this same manner. In Table I, calli derived from three independently transformed plants Tr12, Tr24, and Tr31, each transformed with the construction pRT45 (31), were examined for their induction on auxin-less media. All three of these transformed lines were induced to high levels on the basal media. The levels of induction ranged from 15- to 50-fold higher than the levels of CAT protein induced in whole, wounded plants. The transformant which is most highly inducible by wounding (Tr24) is also most inducible by auxin depletion. In addition, three other transformants, Tr18, Tr19, and Tr62, were also tested. These plant lines were transformed with pRT50, a derivative of pRT45 in which the inhibitor II transcriptional terminator is replaced with the terminator from transcript 7 of *Agrobacterium tumefaciens* T-DNA (31). Plants transformed with this construction are drastically reduced in their response to wounding. Calli derived from these plants do not express CAT protein in the absence of auxin (Table I). Finally, one additional cell line was tested, construction pRT45 was transferred into *Nicotiana tabacum* cv Xanthi using a wild-type *Agrobacterium* vector, A281. The *pin2*-CAT gene in this cell line was not inducible by plating onto auxin-free medium.

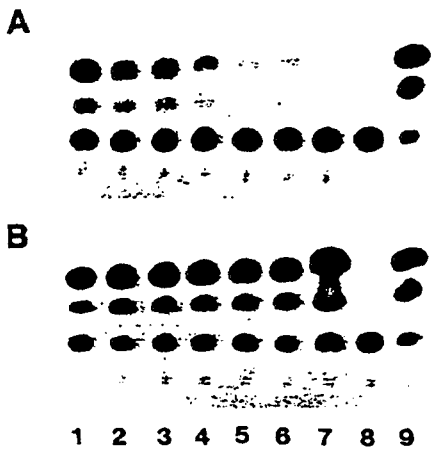

Figure 2. Expression of CAT activity in calli from Tr12 on media containing various hormone levels. Calli were incubated for 7 d on the various media and then processed for CAT assay as described in "Materials and Methods." A, Addition of NAA to the basal medium; B, addition of BAP to the basal medium. Lane 1, no addition; lane 2, 0.02 mg/L; lane 3, 0.05 mg/L; lane 4, 0.2 mg/L; lane 5, 0.5 mg/L; lane 6, 2.0 mg/L; lane 7, 10 mg/L, lane 8, negative control; lane 9, positive control containing 0.1 unit of purified *Escherichia coli* CAT enzyme. Control levels of CAT activity were 55% conversion of chloramphenicol into chloramphenicol acetates.

Since auxins appear to regulate the expression of this chimeric *pin2*-CAT gene fusion in transgenic tobacco tissues analogous to wounding of whole plants, we examined the regulation of this gene by other endogenous and exogenous auxins. As shown in Table II, auxin-less media permitted the activation of the wound-inducible CAT gene fusion in Tr12 calli to a high level. NAA at 2 mg/L (1.2×10^{-5} M) effectively blocked the expression of CAT activity. However, β-NAA was ineffective in repressing the expression of the wound-inducible CAT gene activity. This correlates with previous published reports that β-NAA is not an active auxin (28). Endogenous auxins such as IAA, IPA, IBA, or IAN all repressed the expression of the wound-inducible CAT gene fusion but not as effectively as NAA. The synthetic auxin analogs 2,4-D and 2,4,5-T were approximately 10-fold better than the endogenous auxin, IAA, in repressing the expression of the CAT activity.

Other hormones were also tested for their effect on the expression of the *pin2*-CAT gene in transgenic callus. None of the other hormones tested were able to induce the gene when the hormones were added to maintenance medium; however, when added to the basal medium, ethylene (ethophon, 2-chloroethyl phosphonic acid) increased the level of CAT expression by 25 to 50%, even at the lowest concentrations tested (1.4×10^{-7} M). The nature of this ethylene stimulation has not been further investigated, but since ethylene is produced following wounding, this may be an amplification system to super-stimulate the levels of wound-inducible gene expression. Abscisic acid (at concentrations up to 3.8×10^{-5} M) had no effects upon the action of the wound-inducible *pin2*-CAT gene and gibberellic acid had inhibitory effects at very high concentrations (2.9×10^{-5} M).

Auxin Regulates *pin2*-CAT Expression *in Vivo*

To determine whether this auxin-dependent regulation of the chimeric *pin2*-CAT gene fusion in transgenic tobacco tissues was also operating in whole plants, we examined CAT gene activity in whole plant tissues by a leaf disc assay system. Our initial experiments with leaf discs did not respond to auxin. This was apparently due to the inability of auxin to penetrate the leaf epidermis. When dimethyl sulfoxide is

Table I. *Inducibility of Various Transformed Plants and Tissues*

Construction	Transformant	Conversion[a]		Inducibility	Conversion[b]
		Unwounded	Wounded		Auxin Effect
		%			%
pRT45	Tr12	0.4 ± 0.1	0.8 ± 0.3	2.0	38.4 ± 13.0
pRT45	Tr24	0.5 ± 0.2	3.8 ± 2.8	9.6	59.9 ± 17.3
pRT45	Tr31	0.8 ± 0.4	1.6 ± 1.3	2.0	25.5 ± 12.4
pRT45	NT-RT45-01[c]				1.4 ± 0.6
pRT50	Tr18	0.3 ± 0.1	0.3 ± 0.1	1.0	0.5 ± 0.1
pRT50	Tr19	0.4 ± 0.3	0.4 ± 0.2	1.0	1.1 ± 0.3
pRT50	Tr62	0.6 ± 0.5	0.6 ± 0.4	1.0	0.5 ± 0.1

[a] Wound-induced expression of the CAT gene in transgenic tobacco plants transformed with either pRT45 or pRT50 (see ref. 31 for details of transformation and plasmid construction). Several clonal propagants of each transformant were assayed by the previously described wound assay (31). The number of each wounded transformed plant compiled to yield these data are: Tr12, $n = 5$; Tr24, $n = 9$; Tr31, $n = 6$; Tr18, $n = 5$; Tr19, $n = 7$; Tr62, $n = 4$. The data is presented as the mean ± SD of the % conversion of [^{14}C]chloroamphenicol into [^{14}C]chloramphenicol acetates by 100 μg of protein from an unwounded control leaf and 24-h wounded leaf. The inducibility represents the ratio of the means of % conversion in the wounded leaf to the unwounded leaf of the same plant. [b] Calli derived from each of the transformed plants were plated on media lacking auxin and after 7 d were assayed for CAT activity. The data presented are the means ± SD of the % conversion of [^{14}C]chloramphenicol into [^{14}C] chloramphenicol acetates by 100 μg of protein from the calli; $n = 4$. [c] A cell line derived from transformation of *N. tabacum* cv Xanthi with a wild type *Agrobacterium*, A281; $n = 6$.

Table II. *Effect of Auxins on pin2-CAT Gene Expression*

Auxin	Concentration	Conversion[a]
	μg/mL	%
None		83.2 ± 3.0
α-NAA	2	4.5 ± 2.4
β-NAA	2	84.7 ± 7.2
IAA	2	9.9 ± 4.1
IPA	2	17.0 ± 10.2
IBA	2	17.6 ± 13.7
IAN	2	10.1 ± 2.0
2,4-D	0.2	29.0 ± 10.6
2,4,5-T	0.2	6.2 ± 2.6

[a] Conversion of [^{14}C]chloramphenicol to [^{14}C]chloramphenicol acetate by 100 μg of extracted plant protein. Data presented are the mean ± SD; $n = 4$. All auxin values except β-NAA differ significantly from control at the 99.9 confidence level.

included at 2 ppm (Fig. 3, lanes 1–3) to facilitate cell entry, the auxin does indeed down regulate *pin2*-CAT gene expression; however, high concentrations were required to completely turn off gene expression suggesting that this procedure may not allow full equilibration of auxin across the epidermis. When the epidermis was removed, lanes 4 to 6, lower levels of auxin (<10 μg/mL) were required to completely repress activity of the wound-inducible chimeric gene in whole plant tissues.

When leaf discs were incubated in MS media containing cytokinin alone, the *pin2*-CAT gene was still induced to high levels (not shown). Since cytokinin alone can maintain the viability of solanaceous leaf tissue, this suggests that viable leaf tissue is responding to the absence of auxins in these whole plant tissues.

DISCUSSION

Transgenic tobacco callus containing a wound-inducible proteinase inhibitor II-CAT gene fusion is regulated in callus

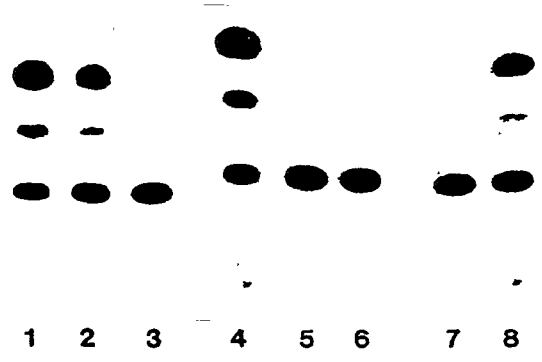

Figure 3. Expression of CAT activity in leaf discs from a Tr25 plant incubated in MS liquid medium alone or with the addition of NAA. Lanes 1 to 3 contain 2 ppm dimethyl sulfoxide to aid penetration of the epidermis by the auxin. Leaf discs in lanes 4 to 6 have the epidermis removed prior to incubation. Leaf discs were incubated for 18 h in the MS media and then processed for CAT assay as described in "Materials and Methods." CAT assays were performed with 100 μg of total leaf protein. Lanes 1 and 4, no addition; lanes 2 and 5, 10 mg/L NAA; lanes 3 and 6, 100 mg/L NAA; lanes 7 and 8 are negative and positive controls, respectively, containing either no addition or 0.1 unit of purified *E. coli* CAT enzyme.

tissues and in leaf discs by exogenously applied auxin. Other hormones had either little or slight stimulatory effects on the expression of *pin2*-CAT gene in the absence of auxin. This auxin effect is specific and has been shown to function in several independently derived wound-inducible transformants. Levels of CAT expression in transgenic callus maintained on basal medium correlated to the wound-inducible expression found in whole plants. Plants which showed the highest levels of wound-inducible expression of CAT protein also showed the greatest turn-on in the absence of auxin in

callus. The standard deviation of these auxin studies was frequently high and several factors may contribute. The size of the explant may be one of these factors as well as the nature of cut surfaces on the explant.

Lines of transgenic callus derived from plants which contain a noninducible derivative of pRT45 are not affected by auxin. Additionally, a cell line derived from transformation of tobacco with wild-type *Agrobacterium tumefaciens* vectors did not induce the *pin2*-CAT gene by plating onto hormone free media. Such transformed calli frequently contain independently cotransformed T-DNA sequences, along with the gene of interest (7). This cell line has been maintained on media without hormones or on regeneration media (MS plus 0.5 BAP) for more than 2 years, yet it has never regenerated plants. Thus, this cell line NT-RT45-01 likely contains the wild-type T-DNA locus as well as the pRT45 locus. The T-DNA locus contains a well characterized pathway for the production of IAA in the plant tissues (30). This pathway aids in maintaining the callus in the undifferentiated state (27). Presumably, since these calli produce their own auxin, this cell line, NT-RT45-01, is not induced by plating these calli onto the basal media lacking auxin. We did not, however, determine the endogenous levels of auxin in the NT-RT45-01 calli.

Whether changes in auxin homeostasis permits other plant defense genes to be induced is not known; however, exogenously applied auxins have been shown to repress invertase in Jerusalem artichoke tuber slices (8), peroxidases in young peas (22), and another potato proteinase inhibitor (25). In addition, exogenously applied auxins have been reported to induce susceptibility of maize to *Helminthosporium* leaf spot (15) and of tobacco to tobacco mosaic virus (29). Thus, auxin fluxes may also play a role in the regulation of several plant defense genes.

Further, since increasing auxin down-regulates this plant defense gene, it is possible that this may play an important role in plant pathogenesis by microorganisms. Many plant pathogens endogenously produce auxin (20) and this auxin is thought to be a virulence factor for some pathogens (27). Production of sufficient amounts of auxin in the infected tissue could effectively block the expression of this or other auxin-repressible plant defense genes.

LITERATURE CITED

1. Benveniste I, Salaun J-P, Durst F (1977) Wounding-induced cinnamic acid hydroxylase in Jerusalem artichoke tuber. Phytochemistry 16: 69–73
2. Bishop PD, Makus DJ, Pearce G, Ryan CA (1981) Proteinase inhibitor inducing factor activity in tomato leaves resides in oligosaccharides enzymatically released from cell walls. Proc Natl Acad Sci USA 78: 3536–3540
3. Boller T, Gehri A, Mauch F, Vögeli U (1983) Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. Planta 157: 22–31
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 72: 248–254
5. Cleveland TE, Thornburg RW, Ryan CA (1987) Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol Biol 8: 199–207
6. Corbin DR, Sauer N, Lamb CJ (1987) Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. Mol Cell Biol 7: 4337–4344
7. De Framond AJ, Back EW, Chilton WS, Kayes L, Chilton MD (1986) Two unlinked T-DNAs can transform the same tobacco plant cell and segregate in the F_1 generation. Mol Gen Genet 202: 125–131
8. Edelman J, Hall MA (1964) Effect of growth hormones on the development of invertase associated with cell walls. Nature 201: 296–297
9. Fox EA (1986) Isolation and characterization of a proteinase inhibitor II gene from *Lycopersicon esculentum*. Doctoral Dissertation, Washington State University, Pullman, WA
10. Gorman CN, Moffat LF, Howard BH (1982) Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. Mol Cell Biol 2: 1044–1051
11. Graham JS, Hall G, Pearce G, Ryan CA (1986) Regulation of synthesis of proteinase inhibitors I and II mRNAs and leaves of wounded tomato plants. Planta 169: 399–405
12. Graham J, Pearce G, Merryweather J, Titani K, Ericsson L, Ryan CA (1985) Wound-induced proteinase inhibitor from tomato leaves. II. The cDNA deduced primary structure of pre-inhibitor II. J Biol Chem 260: 6561–6564
13. Green TR, Ryan CA (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777
14. Gustafson G, Ryan CA (1976) The specificity of protein turnover in tomato leaves: the accumulation of proteinase inhibitors induced with the wound hormone, PIIF. J Biol Chem 251: 7004–7010
15. Hoffman SE, Zscheile FP (1973) Leaf bioassay for *Helminthosporium carbonum* toxin-search for phytoalexin. Phytopathology 63: 729–734
16. Keil M, Sanchez-Serrano J, Schell J, Willmitzer L (1986) Primary structure of a proteinase inhibitor II gene from potato (*Solanum tuberosum*). Nucleic Acids Res 14: 5641–5650
17. Lawton MA, Lamb CJ (1987) Transcriptional activation of plant defence genes by fungal elicitor, wounding, and infection. Mol Cell Biol 7: 335–341
18. Lee JS, Brown WE, Graham JS, Pearce G, Fox EA, Dreher TW, Ahern KG, Pearson GD, Ryan CA (1986) Molecular characterization and phylogenetic studies of a wound-inducible proteinase inhibitor I gene in *Lycopersicon* species. Proc Natl Acad Sci USA 83: 7277–7281
19. Maule AJ, Ride JP (1976) Ammonialyase and *O*-methyl transferase activities related to lignification in wheat leaves infected with *Botrytis*. Phytochemistry 15: 1661–1664
20. Morris RO (1986) Genes specifying auxin and cytokinin biosynthesis in phytopathogens. Annu Rev Plant Physiol 37: 509–538
21. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
22. Ockerse R, Siegel BI, Galston AW (1966) Hormone-induced repression of a peroxidase isozyme in plant tissue. Science 151: 452–453
23. Pena-Cortez H, Sanchez-Serrano J, Rocha-Sosa M, Willmitzer L (1988) Systemic induction of proteinase inhibitor II gene expression in potato plants by wounding. Planta 174: 84–89
24. Ryan CA, Bishop P, Pearce G, Darvill AG, McNeil M, Albersheim P (1981) A sycamore cell wall polysaccharide and a chemically related tomato leaf polysaccharide possess similar proteinase inhibitor-inducing activities. Plant Physiol 68: 616–618
25. Ryan CA (1968) Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. Plant Physiol 43: 1859–1865
26. Sacher JA, Roshalda-Williams JT, Cabello A (1982) Wound-induced RNase activity in sweet potato. Plant Physiol 69: 1060–1065
27. Sachs T (1975) Plant tumors resulting from unregulated hormone synthesis. J Theor Biol 55: 445–453
28. Schneider EA, Wightman F (1978) Auxins. *In* DS Letham, PB Goodwin, TJV Higgins, eds, Phytohormones and Related

Compounds—A Comprehensive Treatise. Vol 1. Elsevier, New York. pp 29–105
29. Simons TJ, Israel HW, Ross AF (1972) Effect of 2,4-dichlorophenoxyacetic acid on tobacco mosaic virus lesions in tobacco and on the fine structure of adjacent cells. Virology 48: 502–515
30. Thomashow LS, Reeves S, Thomashow MF (1984) Crown gall oncogenesis: evidence that a T-DNA gene from *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. Proc Natl Acad Sci USA 81: 5071–5075
31. Thornburg RW, An G, Cleveland TE, Johnson R, Ryan CA (1987) Wound-inducible expression of a potato inhibitor. II—chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. Proc Natl Acad Sci USA 84: 744–748

Plant Physiol. (1991) 96, 802–805
0032-0889/91/96/0802/04/$01.00/0

Received for publication December 18, 1990
Accepted March 28, 1991

Wounding *Nicotiana tabacum* Leaves Causes a Decline in Endogenous Indole-3-Acetic Acid[1]

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ABSTRACT

We have previously observed that auxin can act as a repressor of the wound-inducible activation of a chimeric potato proteinase inhibitor II-CAT chimeric gene (*pin2*-CAT) in transgenic tobacco (*Nicotiana tobacum*) callus and in whole plants. Therefore, this study was designed to examine endogenous levels of indole-3-acetic acid (IAA) in plant tissues both before and after wounding. Endogenous IAA was measured in whole plant tissues by gas chromatography-mass spectrometry using an isotope dilution technique. ^{13}C-Labeled IAA was used as an internal standard. The endogenous levels of IAA declined two- to threefold within 6 hours after a wound. The kinetics of auxin decline are consistent with the kinetics of activation of the *pin2*-CAT construction in the foliage of transgenic tobacco.

When plant foliage is mechanically wounded a series of specific and nonspecific plant defenses are induced (9, 14). One group of the well studied defense products are the proteinase inhibitors of solanaceous plants. These proteinase inhibitors are activated at the transcriptional level following wounding (13). However, their kinetics of induction (mRNA begins to appear 2 to 4 h after wounding) makes them distinct from other wound-inducible genes from the lignin and phytoalexin biosynthetic pathways (9). Genes encoding the proteinase inhibitors from both tomato and potato have been isolated and characterized (6, 12, 19, 23, 27).

Transgenic plants have been made with both proteinase inhibitor I and II and the expression of these genes in plants has been well studied (15, 23, 27). When tobacco plants bearing the *pin2*-CAT gene were examined for their expression of CAT protein, they expressed CAT protein in a manner which was identical with the wild-type expression of proteinase inhibitor II in young tomato plants (26, 28). Therefore, these plants make a good model system for the study of the wound-inducible genes. Further, when introduced into potato, the same chimeric gene confers both wound-induced and tuber specific expression on the transgenic plants (17).

Because these transgenic plants are a good model system for the study of wound-inducible genes, we have previously investigated the role of the five classical plant hormones on the induction of the *pin2*-CAT gene. These studies revealed that auxin, when present at near physiological levels, can repress the synthesis of CAT protein in the transgenic callus and also in plants (18).

This specific repression of the inhibitor II gene activation led us to address the question of IAA levels in whole plants following wounding. In this study we have measured the levels of endogenous IAA in the foliage of whole tobacco plants by an isotope dilution technique coupled with a GC-MS detection system.

MATERIALS AND METHODS

Plants

The plants used in this study were tobacco, *Nicotiana tabacum* cv Xanthi. Both wild-type and transformed progeny plants were used. The transformed plants were previously described (28). They were an R4 homozygous line of transgenic plants (four self-pollinations after regeneration, R0) containing a *pin2*-CAT construction which was also previously described (27). All plants were used once and then never again, so that multiple wounds did not occur on the same plant.

Materials

[^{13}C]IAA (3a,4.5,6,7,7a-hexa[^{13}C]indole-3-acetic acid) was a gift of Dr. Jerry D. Cohen (USDA Agricultural Research Center) to Dr. C. E. LaMotte of the Iowa State University Plant Hormone Analysis Facility. Unlabeled IAA was from Sigma Chemical Co. Butylated hydroxytoluene was obtained from Fisher Chemical Co. Diazomethane was generated according to the method of Feiser and Feiser (11) and used within 2 months of generation. The diazomethane was stored in 50 mL aliquots at −20°C. All other materials were obtained locally.

Wounding

Only large fully expanded leaves of the transgenic tobacco plants were used in this study. The leaves were wounded by repeated pinching with a pair of surgical hemostats which caused a large, severe wound (2 cm wide and extending from the leaf edge to the midvein) across the central portion of the leaf as previously described (28). To avoid interplant and interleaf variability in the IAA assays, we utilized a half-leaf assay. In this assay, one-half of the leaf lamina (from one edge up to but not including the midvein) was removed at the start

[1] This work was supported by grants from the U.S. Department of Agriculture (87-CRCR-1-2518), the State of Iowa, and the Iowa Biotechnology Council.

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of the experiment and immediately frozen in liquid nitrogen. The opposite half-leaf was wounded as above and left on the plant for the incubation period. After the indicated time, the remaining wounded half of the leaf lamina (up to but not including the midvein) was removed and also frozen in liquid nitrogen. Sometimes several leaves from a single plant were processed together by this half-leaf assay to provide sufficient material for analysis. The tissues were stored at −70°C until analysis. Both the unwounded sample and the wounded sample from the same plant were processed for IAA analysis on the same day as described below.

CAT assays were conducted as previously described (18) and used to confirm that the wounding process was sufficient to induce this gene system.

IAA Analysis

These analyses were conducted at the Iowa State University Plant Hormone Analysis Facility. IAA analysis was conducted by an isotope dilution technique (20) using the modified methods of Cohen *et al.* (8). Three grams of leaf tissue were used for each assay of IAA in the plant tissues. The leaf tissue previously stored at −70°C, was ground with 3 g of washed sea sand in a mortar and pestle precooled with liquid nitrogen. Ten milligrams of butylated hydroxytoluene per gram of tissue was added during this grinding step. A quantity of acetone (9.6 mL) was added to bring the final concentration of acetone to 80%, assuming that fresh leaves contain 80% water. An additional 20 to 25 mL of 80% acetone was added to facilitate grinding. A known amount of [^{13}C]IAA was added to each sample at the beginning of extraction. Following this extraction, the ground material was rapidly filtered through two layers of Whatman No. 1 paper. The extraction was repeated (2 times, 20 min) with 80% (v/v) acetone:H_2O. The filtrate and washings were pooled into a 100 mL pear-shaped flask and reduced to the aqueous phase by rotary evaporation at 35°C under vacuum. The pH was adjusted to 3.0 with HCl. The IAA was then partitioned into ethyl acetate and the ethyl acetate was removed at 35°C under reduced pressure. A highly viscous, pigmented, oily residue remained. The pigments were removed by dissolving the residue in 15 mL (3 × 5 mL) of 70% methanol and passing the methanolic solution through a C-18 Sep-Pak in small aliquots. The Sep-Pak was prewashed with 10 mL of 100% methanol followed by 10 mL of 70% methanol. The pigments bound to the column, while the IAA eluted freely. The eluents were pooled and roto-evaporated to dryness at 35°C. The residue which contains IAA was dissolved in a total of 1.2 mL of 20% methanol/80% 0.1 M acetic acid. The entire sample was applied to a 1 × 25 cm Phenomenex HPLC column containing a 5 μm Apex octadecyl (C-18) silica. A gradient was developed from 20% methanol/80% 0.1 M acetic acid to 100% methanol. In this gradient IAA elutes between 63 to 73% methanol. Those fractions containing IAA were pooled and concentrated in a roto-evaporation flask to remove the methanol and acetic acid.

The remaining material contained the free IAA. It was dissolved in 50 μL of methanol and methylated with diazomethane for 30 min at room temperature. The partially purified IAA-methyl ester was concentrated to a small volume (10 μL) and 2 μL aliquots were injected into a Hewlett-Packard 5890 gas chromatograph. We utilized a splitless injection so that 100% of the GC effluent was bled into a Hewlett-Packard 5970 quadropole mass spectrometer. This spectrometer utilized an electron impact method to generate ionizations. Ions with mass to charge ratios of 189 and 195 (the molecular ion) and 130 and 136 (the major fragment, IAA minus the carboxymethyl group) were selectively monitored and the data were stored in digital form. GC-MS parameters were set according to Cloud (7). The recovery of IAA by this method was estimated by two methods. With this system, Cloud reported a recovery of approximately 50% using radiolabeled IAA. We have also monitored the total ion count for [^{13}C]IAA. By comparison with ion counts from known amounts of [^{13}C]IAA, we calculate an average recovery of [^{13}C]IAA that ranges from 47 to 67% for the experiments described herein.

Calculations

The amount of IAA in leaves was calculated by the isotope dilution equations (20) as follows:

$$Y = X/R\,[(C_i/C_f) - 1] \qquad (1)$$

where:

Y = amount of naturally occurring IAA (in ng),
X = known amount of [^{13}C]IAA added to sample as internal standard (in ng);
R = the ratio of the fraction of endogenous IAA that has a peak at m/z 130 to the fraction of the internal standard that is fully substituted and has an ion at m/z 136 (8).
C_i = initial concentration (%) of [^{13}C]IAA [before extraction] = 100%
C_f = final concentration (%) of [^{13}C]IAA [after extraction]

$$= \frac{\text{methyl-[}^{13}\text{C]IAA peak area}}{\text{methyl-[}^{13}\text{C]IAA peak area} + \text{methyl-}^{12}\text{C-IAA peak area}} \times 100\%.$$

X/R was always determined in parallel for each set of determinations. A known amount of [^{12}C]IAA (Y) was added to an estimated amount of [^{13}C]IAA (X/R, the exact amount was unknown). The [^{12}C]- and [^{13}C]IAA were mixed and methylated, then processed through GC-MS. X/R was then calculated by rearranging Equation 1 to obtain the following:

$$X/R = \frac{Y}{[(C_i/C_f) - 1]} \qquad (2)$$

Therefore, with the amount of [^{13}C]IAA (X/R) known, the amount of naturally occurring IAA in leaf sample ([^{12}C]IAA) can be calculated with Equation 1.

RESULTS AND DISCUSSION

Tobacco foliage has been previously examined for levels of IAA and found to contain approximately 10 to 20 ng of free IAA per gram of leaf tissue (2). Therefore, to determine whether our IAA assay was sufficiently sensitive to measure

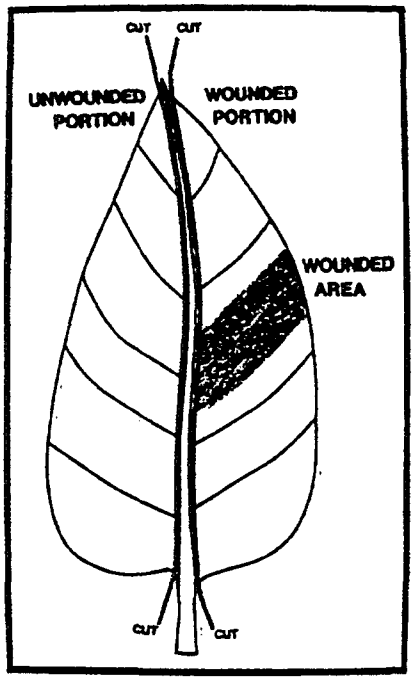

Figure 1. Before wounding with hemostats, one-half of each leaf was removed and immediately frozen in liquid nitrogen for a time zero control. At various times postwounding, the remaining leaf blade halves minus the central midvein were harvested, frozen, and analyzed on the same day as the time zero samples.

these low concentrations of IAA in tobacco leaf tissues, we constructed a calibration curve by adding a standard amount of [^{13}C]IAA to samples containing varying amounts of [^{12}C] IAA ranging from 2 to 30 ng. These samples were extracted and IAA isolated according to the method outlined above. [^{12}C]- and [^{13}C]IAA was quantitated with the GC-MS assay. We obtained a linear standard curve over the range of interest, and we were able to accurately detect low amounts (2 ng) of IAA. Thus, the isolation and quantitation of IAA in our hands was sufficiently sensitive to determine the amounts of IAA in tobacco foliage.

Our initial studies to measure IAA in plant tissues found high variability in the levels of IAA in different leaves. This variability may relate to the positioning of the leaf on the plant as has been previously observed (24); however, even among adjacent leaves, we observed sufficiently high variation in IAA levels of the unwounded state that we had little confidence in the reproducibility of the assay when different leaves were used for the wounded and unwounded samples. Because of this variability, we began to use an assay in which half of the leaf was removed for a time zero control, and the other half of the leaf was treated to determine wounded levels of IAA (Fig. 1).

When transgenic plants were assayed, a small portion of the wounded and unwounded leaf material was utilized for CAT assays to determine the activity of the *pin2*-CAT gene. Routinely, we found that in the time zero sample there was no CAT activity, after 3 h there was low level of induction, and at later timepoints, CAT activity was well induced (28). Because we found that there are significant differences in IAA content of unwounded leaves between plants and among different leaves on the same plant, the data are expressed as percent decline in IAA content in the wounded halves of leaves relative to the unwounded leaf halves. Data expressed in this manner utilizing an "internal" time zero control are much more consistent between replicates even though the levels of IAA in unwounded leaves varies between replicates.

These analyses indicate that the levels of IAA found in the foliage declines following wounding of the foliage (Table I). This decline is maximal between 3 and 6 h after the wound. Eventually, the levels of IAA decline by two- to threefold in the 24 h following the wound. The kinetics of this decline in IAA levels is very similar to the kinetics of activation of the proteinase inhibitor gene system in either normal tomato plants (13), or in transgenic tobacco plants (28). This decline in IAA levels in wounded tobacco foliage is consistent with a role of IAA in the regulation of the inhibitor II gene system.

In other systems, such as sweet potato tubers, IAA levels are low in unwounded tissues (1–5 ng/g fresh weight) and increase dramatically following wounding reaching a maximum after 18 h (25). This observation raises an interesting point about the tuber specific regulation of proteinase inhibitors. In *Solanum tuberosum* tubers the proteinase inhibitor genes are normally expressed at high levels, then following wounding, transcription of the inhibitor genes are shut off (17). While sweet potato tubers arise from distinctly different structures than potato tuber tissue, this finding does raise the question of whether IAA can also participate in the regulation of expression of the inhibitor genes in potato tubers as well as in the foliage.

Other investigators have implicated a variety of other biochemical compounds in the regulation of the inhibitor II gene system. Among these compounds are endogenous plant cell wall oligosaccharide fragments (3, 4), fungal cell wall fragments (29), sucrose (16), abscisic acid (21), methyl jasmonate

Table I. *Levels of IAA following Wounding of Tobacco Leaves*

Incubation Time	Unwounded	Wounded	Percentage of Control[b]
h	*ng IAA per g fresh wt*[a]		
0	18.9 ± 2.0	18.6 ± 3.0	98.1 ± 7.5
3	10.5 ± 0.5	8.8 ± 1.1	84.3 ± 9.0
6	12.7 ± 3.4	8.2 ± 0.2	68.0 ± 17.9
12	18.4 ± 7.6	8.9 ± 2.6	54.9 ± 28.1
24	16.2 ± 5.9	7.5 ± 3.9	44.4 ± 8.0

[a] One-half of the leaf lamina (from one edge up to but not including the midvein) was removed at time 0 and immediately frozen in liquid nitrogen. The opposite half was wounded by repeated pinching with a pair of surgical hemostats in a 2 cm wide band of tissue. These wounded half-leaves were incubated on the plant for the indicated times, and then the wounded half of the leaf lamina (up to but not including the midvein) was removed and frozen in liquid nitrogen. For each time point, the two samples were processed sequentially and analyzed on the same day. [b] Data is expressed as percentage of IAA present in the wounded leaf halves divided by that present in the control unwounded leaf halves ($n = 3$).

(10), and IAA (18). All of these compounds, with the exception of IAA, have inducing effects on the expression of the inhibitor II gene system, whereas IAA represses the expression of the genes. We are currently trying to determine the hierarchy of the inductive and repressive effects played by these compounds in inducing the inhibitor II gene within the plant.

Further, we are interested in determining the effect that each of these positive regulating compounds has on the levels of IAA within the plant. There are several reports (1, 5, 22) that exogenous application of ABA to plant tissues can cause a decrease in the endogenous levels of IAA within those tissues. These effects are thought to occur through either influencing auxin transport or through direct effects on the auxin metabolism. Thus, some of the many positive regulating compounds indicated above, especially ABA, may act to induce inhibitor gene activation by decreasing the levels of IAA within the responding tissues. Experiments to examine this are currently underway.

Our working hypothesis is that endogenous levels of IAA in unwounded plant tissues are sufficient to maintain the inhibitor II gene system in a repressed state so that the genes are not expressed. However, following a wound, the levels of IAA in bulk tissues decline by two- to threefold, allowing a derepression of the gene system with concomitant expression of the CAT protein. The exact role of IAA in the regulation of the inhibitor II gene system is unknown and will require further elucidation. The roles of the other positive regulators will also need to be determined within the framework of the IAA derepression of the inhibitor II gene system.

Also, the mechanism which is responsible for the decline in IAA levels is not yet understood. The IAA pool size could be influenced at one of several points, including the biosynthesis of IAA, its degradation, or the formation of amide or ester storage forms of IAA. Indeed, the decrease in IAA pools in leaves could even be influenced through IAA transport.

ACKNOWLEDGMENT

The authors would like to thank Dr. C. E. LaMotte for his expertise in hormone analysis and his aid in initiating these experiments.

LITERATURE CITED

1. Anker L (1975) Auxin-synthesis inhibition by abscisic acid, and its reversal by gibberellic acid. Acta Bot Neerl 24: 339–347
2. Bandurski RS, Schultze A (1974) Concentrations of indole-3-acetic acid and its esters in *Avena* and *Zea*. Plant Physiol 54: 257–262
3. Bishop P, Pearce G, Bryant J, Ryan C (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. J Biol Chem 259: 13172–13177
4. Bishop PD, Makus DJ, Pearce G, Ryan CA (1981) Proteinase inhibitor-inducing factor activity in tomato leaves resides in oligosaccharides enzymatically released from cell walls. Proc Natl Acad Sci USA 78: 3536–3540
5. Chang Y-P, Jacobs WP (1973) The regulation of abscission and IAA by senescence factor and abscisic acid. Am J Bot 60: 10–16
6. Cleveland T, Thornburg RW, Ryan CA (1987) Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol Biol 8: 199–207
7. Cloud NP (1987) *Gas chromatographic mass spectrometric determinations of indole-3-acetic acid concentrations in lateral* and terminal buds of *Phaseolus vulgaris* and their relationship to apical dominance. MS thesis. Iowa State University, Ames
8. Cohen JD, Baldi BG, Slovin JP (1986) $^{13}C_6$-[Benzene ring]-indole-3-acetic acid: a new internal standard for quantitative mass spectral analysis of indole-3-acetic acid in plants. Plant Physiol 80: 14–19
9. Dixon RA (1986) The phytoalexin response: elicitation, signalling and control of host gene expression. Biol Rev 61: 239–291
10. Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA 87: 7713–7716
11. Feiser FL, Feiser M (1967) Reagents for Organic Synthesis. John Wiley & Sons, New York
12. Fox EA (1986) Isolation and characterization of a proteinase inhibitor II gene from *Lycopersicon esculentum*. PhD dissertation, Washington State University, Pullman
13. Graham JS, Hall G, Pearce G, Ryan CA (1986) Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169: 399–405
14. Green T, Ryan C (1972) Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 175: 776–777
15. Johnson R, Lee JS, Ryan CA (1990) Regulation of expression of a wound-inducible tomato inhibitor I gene in transgenic nightshade plants. Plant Mol Biol 14: 349–356
16. Johnson R, Ryan CA (1990) Wound-inducible potato inhibitor II genes: enhancement of expression by sucrose. Plant Mol Biol 14: 527–536
17. Keil M, Sanchez-Serrano J, Willmitzer L (1989) Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. EMBO J 8: 1323–1330
18. Kernan A, Thornburg RW (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II—chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol 91: 73–78
19. Lee J, Brown W, Graham J, Pearce G, Fox E, Dreher T, Ahern K, Pearson G, Ryan C (1986) Molecular characterization and phylogenetic studies of a wound-inducible proteinase inhibitor I gene in *Lycopersicon* species. Proc Natl Acad Sci USA 83: 7277–7281
20. Magnus V, Bandurski RS, Schultze A (1980) Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for using in mass spectrometric assays. Plant Physiol 66: 775–781
21. Pena-Cortez H, Sanchez-Serrano JJ, Mertens R, Willmitzer L, Prat S (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc Natl Acad Sci USA 86: 9851–9855
22. Pilet PE, Rebeaud JE (1983) Effect of abscisic acid on growth and indoyl-3-acetic acid levels in maize roots. Plant Sci Lett 31: 117–122
23. Sanchez-Serrano JJ, Keil M, O'Connor A, Schell J, Willmitzer L (1987) Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants. EMBO J 6: 303–306
24. Schneider EA, Wightman F (1978) Auxins. *In*. DS Letham, PB Goodwin, TJV Higgins, Phytohormones and Related Compounds: A Comprehensive Treatise. Elsevier, Amsterdam, pp 29–105
25. Tanaka Y, Uritani I (1979) Polar transport and content of indole-3-acetic acid in wounded sweet potato root tissues. Plant Cell Physiol 20: 1087–1096
26. Thornburg RW, An G, Cleveland TE, Ryan CA (1987) Characterization and expression of a wound-inducible proteinase inhibitor II gene from potato. NATO ASI (Adv. Sci. Inst.) Sr. Ser. A. Basic Life Sci 140: 121–129
27. Thornburg RW, An G, Cleveland TE, Johnson R, Ryan CA (1987) Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. Proc Natl Acad Sci USA 84: 744–748
28. Thornburg RW, Kernan A, Molin L (1990) CAT protein is expressed in transgenic tobacco in field tests following attack by insects. Plant Physiol 92: 500–505
29. Walker-Simmons M, Ryan CA (1984) Proteinase inhibitor synthesis in tomato leaves: induction by chitosan oligomers and *chemically modified chitosan and chitin*. Plant Physiol 76: 787–790